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TEMPERATURE COEFFICIENT OF THE ACTION OF β-RAYS UPON THE EGG OF NEREIS.

By ALFRED C. REDFIELD and ELIZABETH M. BRIGHT.

(From the Laboratory of Physiology, Harvard Medical School, Boston, and the Marine Biological Laboratory, Woods Hole.)

(Received for publication, November 4, 1918.)

Lepper[1] has shown that grafts of rat sarcoma radiated at 15°C. show very little difference in their behavior from those radiated at 37°C., but that the latter grow rather more slowly and disappear sooner than those which have been radiated in the cold. So far as we are aware no one has determined the temperature coefficient of the physiological processes, nor of the purely chemical reactions produced by rays from radioactive substances. The velocity of photochemical processes, to which these reactions are closely allied, is known to be affected very slightly by the temperature at which the processes occur. The great mass of physiological reactions shares a high temperature coefficient (2 to 3 for a change of 10°C.) with general, non-photochemical reactions.[2] Data on the temperature coefficient of photosensory stimulation in animals do not appear to have been published. Dr. Selig Hecht has recently determined that the temperature coefficient of the sensitization process in Mya is of the order of that of a photochemical reaction (personally communicated results of unpublished experiments). Brown and Heise,[3] reviewing the literature of this aspect of photosynthesis, conclude that this important photophysiological process has a temperature coefficient of the same order (1.1 to 1.2 for a change of 10°C.) as those of photochemical reactions, although the investigators who secured the data on which this conclusion is based had assigned higher values to this

[1] Lepper, E. H., *Arch. Middlesex Hospital*, 1914, xxxiii, 77.

[2] Kanitz, A., Die Biochemie in Einzeldarstellungen; Temperature und Lebensvorgänge, Berlin, 1915, pt. 1.

[3] Brown, W. H., and Heise, G. W., *Philippine J. Sc., C*, 1917, xii, 1.

constant. Osterhout and Haas,[4] who likewise obtain a high value, 1.81, for the temperature coefficient of photosynthesis, attribute it to the fact that they are dealing with a series of catenary reactions, of which the determining member is not a true photochemical process.

The development of a method of measuring the physiological action of radiations from radium has enabled us to determine the effect of temperature upon this process with precision. This method depends upon the fact, first observed by Packard,[5] that the fertilization membrane of the egg of the marine worm, Nereis, is greatly enlarged if the eggs have been exposed to radium prior to fertilization. We have shown[6] that the extent of this change is a reliable measure of the intensity of radiation and time of exposure. Our problem has been to determine what effect the temperature at which radiation takes place has upon the velocity of the reaction under uniform intensity.

To this end a few drops of Nereis eggs were placed in the bottom of a test-tube and this was inserted into a vessel containing chopped ice and allowed to stand until it had taken on the temperature of the ice. A tube of radium emanation was then suspended above the eggs. As radiation proceeded, a few eggs were withdrawn from time to time, placed in sea water at room temperature, fertilized, and the thickness of the membranes was measured after a uniform period of time. A second lot of eggs from the same female was then treated in exactly the same way, except that during radiation the test-tube containing them was placed in a thermos flask filled with water at room temperature.

The exposure of unfertilized eggs to these temperatures, without radiation, does not affect the volume of the membrane which is formed upon fertilization, although somewhat higher temperatures may be expected to cause an enlargement of this structure.[7]

This procedure possesses the advantage that the two lots of eggs are kept at different temperatures only during the period of radiation, during which no visible change occurs in these cells. The proc-

[4] Osterhout, W. J. V., and Haas, A. R. C., *J. Gen. Physiol.*, 1918-19, i, 295.
[5] Packard, C., *J. Exp. Zool.*, 1915, xix, 323.
[6] Redfield, A. C., and Bright, E. M., *Am. J. Physiol.*, 1918, xlv, 374.
[7] Just, E. E., *Biol. Bull.*, 1915, xxviii, 1.

ess of membrane formation does not occur until after fertilization. We have found that this process is greatly affected by temperature and have taken care that it should go on at the same temperature in both lots of eggs. Consequently we have avoided the disturbing in-

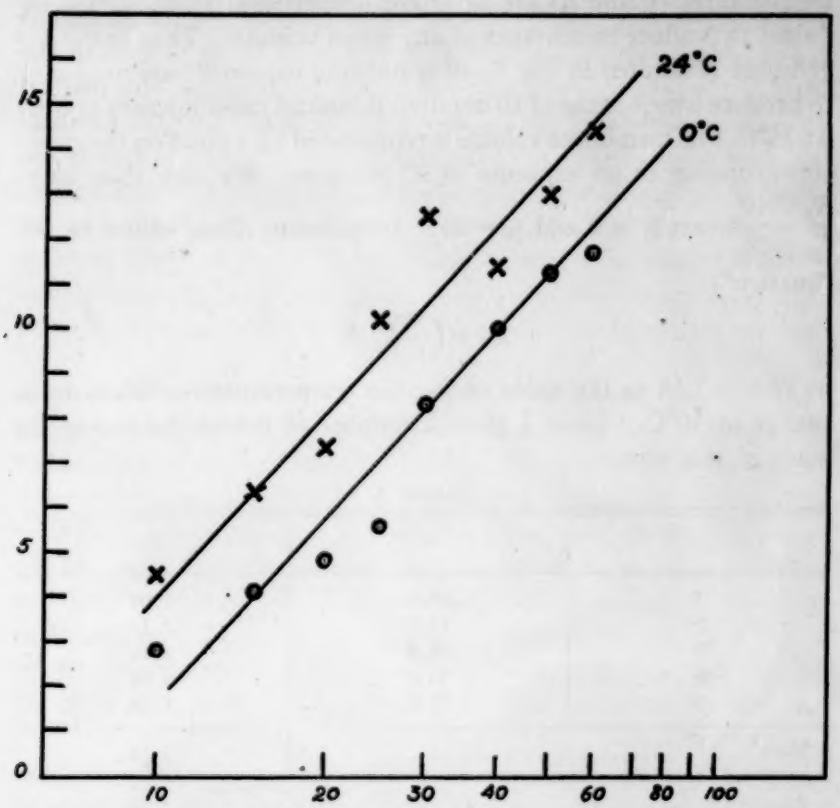

FIG. 1. Curves illustrating the effect of temperature upon the velocity of the change produced by β-rays in the membrane of Nereis eggs. Time of radiation in minutes is measured logarithmically along the abscissa. Volumes of membranes are measured in 100,000 cubic microns along the ordinate. Intensity of radiation was 38.1 millicurie centimeters in both cases.

fluence of a secondary reaction analogous to that to which the high temperature coefficient of photosynthesis is attributable.[4]

When measurements of the volumes of membranes so produced are plotted against the logarithms of the times of exposure, the points

fall about two parallel lines, as illustrated in Fig. 1. The line drawn through the points obtained at the higher temperature lies above the other, showing that the velocity of the reaction has been increased by the higher temperature. The velocities (K_1 and K_2) at the two temperatures (t_1 and t_2) are inversely proportional to the times required to produce membranes of any given volume. Thus in the experiment illustrated in Fig. 1, 40 minutes of exposure were necessary to produce a membrane of 10 hundred thousand cubic microns at 0°C. At 24°C. this membrane volume is represented by a point on the curve corresponding to an exposure of 27 minutes. We may then write $\frac{K_2}{K_1} = \frac{40}{27}$ where $t_1 = 0$ and $t_2 = 24$. Substituting these values in the equation[2]

$$Q_{10} = \left(\frac{K_2}{K_1}\right)^{\frac{10}{t_2 - t_1}}$$

we obtain 1.18 as the value of Q_{10}, the temperature coefficient for a change of 10°C. Table I gives a number of determinations of Q_{10} made in this way.

TABLE I.

t_1	t_2	Q_{10}
0	20.6	1.08
0	23.9	1.11
0	24.8	1.15
0	23.6	1.18
0	24.0	1.18
Mean		1.14

The source of radiation used in this investigation was radium emanation enclosed in slender, thin-walled glass tubes. The walls of these tubes were sufficiently thick to absorb all the α-radiation. We expect to show in a future publication that the effect of the γ-rays from such a tube is negligible compared to that of the β-rays in producing the reaction. The coefficient which we have determined is therefore that of the action of the β-rays.[3]

[3] We are indebted to Dr. William Duane and to the Director of the Cancer Commission of Harvard University for placing a supply of radium emanation at our disposal.

CONCLUSION.

The temperature coefficient of the action of β-rays from radium upon the egg of Nereis lies between 1.1 and 1.2. This is of a magnitude characteristic of photochemical reactions.

ON THE EXPERIMENTAL PRODUCTION OF EDEMA IN LARVAL AND ADULT ANURA.

By CHARLES F. W. McCLURE.

(From the Laboratory of Comparative Anatomy, Princeton University, Princeton.)

(Received for publication, November 5, 1918.)

Edematous frog larvæ are among the commonest types of abnormalities which can be produced by experimental means. When ova of the two- or four-celled stage are placed in solutions of potassium cyanide, acetone, butyric acid, or ethyl alcohol for from 12 to 24 hours, and then subsequently developed in tap water, an edematous condition of the body frequently, though not always, results. Hertwig[1] has produced such larvæ by exposing early cleavage stages to the influence of radium. An edematous condition of the body also occasionally results when ova are allowed to develop in direct sunlight in the laboratory and may even be met with among the controls. The life of such larvæ is relatively short.

The edematous frog larvæ thus far observed by the writer can be grouped into two main types: Type 1, in which, with the exception of their general edematous condition, the frog larvæ appear externally to be otherwise normally developed; Type 2, in which some marked abnormality, especially of the head region and vascular system, usually accompanies an edematous condition of the body.

All the larvæ referred to in this paper were in the stage at which the pronephros acts as the functional kidney and in which the mesonephros is either not at all developed, or is represented only by its earliest anlage.

An examination of serial sections of a large number of both types of edematous frog larvæ shows that in all cases, and without exception, there is a marked deficiency evident in the development of certain specified tubules in the pronephros. Reconstructions after the method of Born clearly indicate that this deficiency is associated with the

[1] Hertwig, O., *Arch. mikr. Anat., 2te Abt.*, 1911, lxxvii, 1 (see Tables II and III for examples of edematous frog larvæ).

tubules which normally constitute the greater portion of the kidney and which occupy a medial and ventral position. In some larvæ none of these particular tubules had even been developed. In all other cases, in which only their partial development had occurred, the few tubules present were always greatly hypertrophied and were often lined by an exceedingly thin-layered epithelium which closely resembled endothelium in appearance.

The less extensive tubular complex which normally occupies a dorsolateral position in the pronephros and into which the nephrostomal canals directly open was, however, invariably found to be developed to a considerable degree in all the edematous larvæ examined. It was lined by a thick-layered epithelium which is the only epithelium of the pronephros in the writer's experience, in both normal and edematous frog or toad larvæ, that has thus far been observed to absorb colloidal acid dyes. In all cases this dorsolateral tubular complex was found to be much hypertrophied and often bladder-like in appearance and, together with the three nephrostomal canals which open into it, was occasionally found to constitute the only portion of the kidney that had been formed. In such extreme cases the pronephric duct was either a solid structure or was not connected with the kidney at all.

Associated with this general defect in kidney development the lymph sinuses of the body, especially those of the head region, were often found to be greatly distended with fluid. In larvæ of Type 1 in which the edema is generalized, no defect in the development of any organs of the body was evident except that of the pronephric tubules referred to. In some cases (Type 2) the anterior lymph hearts were absent, and other prominent defects in the vascular system met with which need not be mentioned here. The pericardial and cœlomic cavities were often greatly expanded and blebs or blisters of various sizes were occasionally formed under the epidermis. All these cavities were filled with fluid, as could easily be determined in larvæ which had been placed in solutions of colloidal acid dyes.

We know that water and certain colloidal acid dyes[2] are continu-

[2] McClure, C. F. W., On the behavior of *Bufo* and *Rana* toward colloidal dyes of the acid azo group, Memoirs of The Wistar Institute of Anatomy and Biology, No. 8, Philadelphia, 1918.

ously absorbed through the integument in both larval and adult frogs and toads when the latter are allowed to remain in such media, and that the excess of fluid taken into the body in these cases is normally eliminated by the kidneys. Since the water taken into the body by absorption through the integument must necessarily reach the kidneys by way of the vascular system, the possibility suggests itself that the edematous condition of the frog larvæ referred to may be due to the deficiency observed in the development of certain specified tubules of the kidney, or, in other words, to a block in the normal kidney function. In consequence of this an excess of water which is constantly being taken into the body through the integument is retained in the body, since it can no longer be eliminated by the kidneys. That this is actually the case, as far as these particular frog larvæ are concerned, seems to be borne out by experiments on adult frogs and toads in which an edematous condition of the body can be produced by ligation of the ureters.

The distal ends of the ureters in the frog and toad lie in the dorsal wall of the cloaca and open dorsally into the latter independently of the bladder, which opens on the ventral wall of the cloaca. It is therefore not a difficult matter to ligate the ureters by means of artery forceps and small metal clamps, or to pass a ligature around the ureters which can be tied over the urostyle. In such cases neither the bladder nor the outlet of the intestinal canal through the cloaca is interfered with.

Among 53 ligation experiments thus far made by the writer, chiefly on adult toads, the six mentioned in Table I illustrate the range of results obtained. In all cases the toads[3] were kept in water for a considerable time and weighed at intervals before applying the ligature. This was done in order to establish a normal balance, under such conditions, between the absorption of water through the integument and its elimination from the body by the kidneys.

The first figure in each of the columns of Table I (Experiments 1 to 6) indicates the weight of the toad in gm. at the time the ureters were ligated and before the toad was again placed in water. The succeeding figures in each column indicate the observations made at different intervals of time.

[3] *Bufo lentiginosus* Shaw.

TABLE I.

Time.	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0	22.8	30.5	30.9	45.9	53.9	49.1
0.50		32.7				
0.52				51.5		
1.10					54.5	
1.35		34.2				
1.40	23.75			54.5		
2.20				57.0	55.4	
2.43	24.8					
3.35			34.0			
5.05	26.9					
5.45				64.4 (40%)		
6.10	27.6					
6.55						55.9
7.45	28.7					
11.55	31.2					
16.55		50.8 (66.5%)				
17.55					60.3	
19.00					60.9	
20.00					61.7	
20.50					62.7	
23.25			58.6 (89.6%) Removed ligature.			
23.30					68.4	
23.40	37.5 (64.4%)					
24.05					70.5 (30%)	
25.25			47.5			52.2
25.55			43.9			
28.35			37.9			56.1
29.55			35.2			
30.50			32.9			
31.55			30.9			
48.40						68.4 (39.3%)

These experiments should not be confused with the well known experiments of Overton[4] who ligated the cloacal opening but not the ureters. When only the cloacal opening is ligated, the flow of urine through the ureters into the cloaca and bladder is not interrupted. The urine in such cases, as stated by Overton, will fill up the bladder and then back up into the intestinal canal. When the ligature is removed, this urine can be readily expressed from the body through the cloaca. On the other hand, as we shall see in the following experiments, after removal of the ligature from the ureters, no fluid can ordinarily be immediately expressed from the cloaca as, in this case, the excess of fluid contained in the body has been deposited in the tissues and body cavity, and not in the bladder and intestinal canal. The six experiments referred to in Table I were made during the month of August.

Experiment 1.—When killed the toad was in a comatose condition and had gained 64.4 per cent in weight in 23 hours and 40 minutes. On removal of the silk ligature no fluid was passed from the cloaca. A clear fluid containing albumin and weighing 6.4 gm. was removed from the subcutaneous lymph sinuses. A clear fluid weighing 4.9 gm. which threw down a precipitate in nitric acid that disappeared when acetic acid was added, was also removed from the body cavity. An excess of fluid amounting to 3.4 gm. still remained in the tissues after removal of the fluid from the lymph sinuses and body cavity. The lungs were inflated and intensely congested. The blood vessels, especially those of the abdomen, integument, and kidneys were also greatly congested.

Experiment 2.—When killed the toad was partially comatose and had gained 66.5 per cent in weight in 16 hours and 55 minutes. No fluid could be expressed from the cloaca on removal of the small metal clamp with which the ureters had been ligated. A bloody fluid weighing 11.6 gm. was removed from the subcutaneous lymph sinuses and 3.2 gm. of fluid of a similar character were found in the body cavity, leaving an excess of fluid amounting to 5.5 gm. in the tissues of the body after removal of fluid from the lymph sinuses and body cavity. The condition of the lungs and blood vessels was essentially the same as in Experiment 1.

Experiment 3.—In this case the dorsal wall of the cloaca was tightly clamped with an artery forceps, the teeth of which undoubtedly cut deeply into the tissues. After being placed in water the toad gained 89.6 per cent in weight in

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[4] Overton, E., *Verhandl. physik.-med. Ges. Würzburg*, 1904, xxxvi.

the course of 23 hours and 25 minutes, at the end of which time the forceps was removed. The toad was in a comatose condition and a few drops of fluid could be expressed from the cloaca. The toad was not killed, and at the end of 8 hours and 30 minutes, after removal of forceps, had regained its normal weight and was very active.

Experiment 4.—When killed the toad had gained 40 per cent in weight in the course of 5 hours and 45 minutes and was still quite active. In ligating the ureters with a silk ligature which passed under the ureters and over the urostyle, a large blood vessel was punctured. The subcutaneous lymph sinuses of the body contained 7.1 gm. of a clear fluid which coagulated on exposure to air, and the body cavity 4.3 gm. of a fluid in which considerable blood was present. The excess of fluid remaining in the tissues after removal of fluid from the lymph sinuses and body cavity amounted to 7.1 gm. The lungs, kidneys, and subcutaneous blood vessels were intensely congested.

Experiment 5.—In this instance the ureters were compressed by a small metal clamp, the jaws of which pressed tightly upon, but did not cut into the tissues. When the toad was killed it was very weak and had gained only 30 per cent in weight in the course of 24 hours and 5 minutes. On removal of clamp no fluid could be expressed from the cloaca. About 7.7 gm. of a slightly bloody fluid were removed from the subcutaneous lymph sinuses and only 1 gm. of a clear fluid containing albumin was found in the body cavity. The excess of fluid still remaining in the tissues after its removal from the lymph sinuses and body cavity amounted to 7.9 gm. The lungs were greatly expanded and intensely congested. Marked signs of congestion were observed on the abdomen, especially in the region of the pelvis.

Experiment 6.—This illustrates an instance in which a gradual increase in body weight is followed by a slight decrease, and then succeeded by a gradual and progressive increase in body weight which develops into an intense form of generalized edema. In this particular case an artery forceps was used in which the teeth had been filed down so that they would not cut into the tissues, and the pressure exerted on the ureters was also relatively slight. When the toad was killed it had gained only 39.7 per cent in weight in the course of 48 hours and 40 minutes. The slow progress of the edema was probably due to an incomplete ligation of the ureters. On removal of forceps about 2.1 gm. of semifluid excreta were expressed from the cloaca. The fluid removed from the subcutaneous lymph sinuses was clear, coagulated on exposure to air, and amounted to 4.2 gm. The fluid present in the body cavity was extremely bloody and also weighed 4.2 gm. The excess of fluid still remaining in the tissues after its removal from the lymph sinuses and body cavity amounted to 8.8 gm. The lungs, kidneys, left oviduct, mesenteries, and subcutaneous blood vessels were intensely congested.

The toad was weighed twenty-one times in the course of this experiment. Only the more critical data, however, are mentioned in Table I.

The experiments clearly show when the ureters are completely ligated and the toad is placed in water, that an increase in body weight immediately takes place which gradually progresses into an intense form of generalized edema. An analysis of the conditions described need not be given here, further than that the progress of the edema appears to be related to the extent and character of the abrasion produced in the tissues at the point of ligation, as well as upon the state of congestion of the tissues which results from ligation.

On the basis of these observations it does not appear difficult to explain the edematous condition of the frog larvæ mentioned above.

We know that the glomeruli of the pronephros are not directly connected with the renal tubules, as is the case in the meso- and metanephros, but project into the body cavity independently of these tubules in the vicinity of the nephrostomes. The filtrate of the pronephric glomeruli must necessarily, therefore, pass into the body cavity before entering the pronephric tubules by way of the nephrostomes. If, on account of a deficiency in their development, the tubules of the pronephros should be unable to take care of all the glomerular filtrate, or to secrete fluid obtained from the pronephric veins, an excess of fluid which could not be eliminated by the kidneys would necessarily remain in the body cavity and tissues and, in the course of time, would increase in amount so that a typical edematous condition of the body would finally result. Such I believe to be an explanation of the edematous condition observed in these frog larvæ.

Further significance of the above observations lies in the fact that they do not appear to bear out Fischer's[5] view that the cause of edema, in the anura at least, resides in the tissues, and is due to an overproduction or accumulation of acids within the body which causes them to take up water. It has also been pointed out by various authors[6] that this view is untenable on account of the limited variation of the hydrogen ion concentration in the body.

[5] Fischer, M. H., Œdema and nephritis; New York, 2nd edition, 1915.

[6] Henderson, L. J., and Cohn, E. J., *J. Am. Chem. Soc.*, 1918, xl, 857. Crozier, W. J., *J. Biol. Chem.*, 1916, xxiv, 255, 443; 1918, xxxv, 455.

STUDIES ON BIOLUMINESCENCE.

IX. Chemical Nature of Cypridina Luciferin and Cypridina Luciferase.*

By E. NEWTON HARVEY.

(From the Department of Marine Biology, Carnegie Institution of Washington, Washington, and the Physiological Laboratory, Princeton University, Princeton.)

(Received for publication, October 12, 1918.)

INTRODUCTION.

In the fourth paper of this series[1] I have described the production of light by the ostracod crustacean, *Cypridina hilgendorfii*, and some of the properties of the photogenic substances found in this animal. Since then I have obtained a large number of *Cypridinæ* and have been able to carry out investigation along chemical lines on a far larger scale. This paper deals with the characteristics of two of the photogenic substances, luciferin and luciferase, in more detail. In the seventh paper[2] reasons are given for the adoption of Dubois' terms, luciferin and luciferase, which in part correspond to my photophelein and photogenin, and I have described how the oxidation product of luciferin, which I have called oxyluciferin, can be reduced to luciferin again. The chemical change involved in the oxidation of luciferin to oxyluciferin appears to be slight and comparable to the change occurring when the leuco-base of a dye is oxidized to the dye itself. It is therefore not surprising to find that oxyluciferin and luciferin have similar properties, and, as far as I have been able to

* Much of this work was performed in the Zoological Laboratory, Imperial University, Tokyo, and I wish to acknowledge the kindness of Professors Ijima, Yatsu, Watase, and Goto during my stay in the University. I am also deeply indebted to Professor Ishikawa of the Agricultural College, Tokyo, for much assistance in collecting material and express my sincere thanks for his interest in the work.

[1] Harvey, E. N., *Am. J. Physiol.*, 1917, xlii, 318.

[2] Harvey, *J. Gen. Physiol.*, 1918, i, 133.

determine, the properties of oxyluciferin and luciferin are identical, so that what is here recorded regarding luciferin will apply to oxyluciferin also. Unless otherwise specified, luciferin, oxyluciferin, and luciferase refer to these substances obtained from *Cypridina hilgendorfii*.

Method.

The living animals are dried quickly in desiccators over $CaCl_2$ and may then be kept indefinitely in well stoppered bottles containing a few lumps of $CaCl_2$ to remove any dampness in the air contained in the bottle. The *Cypridinæ* are well ground to a powder which lights brilliantly if moistened with water, both of the photogenic substances going into solution. It serves as the raw material for the isolation of luciferin and luciferase. From this material an extract may be prepared with distilled water which contains luciferin, luciferase, all the proteins of the animal soluble in water, salts, and other water-soluble material. The extract, filtered through filter paper, is yellow colored and slightly opalescent and glows for some time. The light finally disappears due to the oxidation of the luciferin. There is no change of color on standing. If the extract solution is not too concentrated and well shaken with air, all the luciferin will be oxidized and luciferase alone of the photogenic substances together with oxyluciferin will remain. Salts and other crystalloidal substances may be removed by dialysis since luciferase does not dialyze. The solution is remarkably stable. I have allowed it to dialyze against running tap water in a Schleicher and Schüll parchment diffusion thimble for 2 weeks and then against distilled water for 1 more week without any marked loss of luciferase. Indeed, solutions of luciferase may stand until they become foul and ill smelling from bacterial decomposition without destruction of the luciferase. Solution may be preserved free of bacterial development with toluene or chloroform for many months, but a slow destruction of the luciferase occurs and at the same time a precipitate forms. The luciferase is present in the colloidal state as it does not pass collodion or parchment paper membranes. A solution prepared in the above manner will be known as crude luciferase solution. Although the luciferase can be purified by repeated precipitation with a va-

riety of substances, it loses strength during the process and the crude luciferase solution is the most powerful that can be obtained.

A similar extract may be prepared with *hot* distilled water which contains one of the photogenic substances, luciferin, all of the proteins of the animal not coagulated by heating, the remaining salts, and other material soluble in hot water. My procedure is to add boiling water directly to the dry powder, boil about 20 seconds, and filter quickly while hot. The filtrate is slightly opalescent, yellow colored, and does not darken on standing. On cooling, this solution often glows faintly but if heated to the boiling point a second time the glow ceases and does not return on cooling. However, its content of luciferin is diminished because some has oxidized to oxyluciferin. This hot water extract of *Cypridinæ* will be spoken of as crude luciferin solution. It gives a bright light with luciferase.

If the hot water extract stands in a shallow dish at room temperature, the luciferin disappears completely in the course of some hours, the time depending on its concentration. This is due to oxidation of the luciferin apart from luciferase, as may be easily determined by keeping the luciferin extract in absence of oxygen. I have kept such a solution for 90 days in a test-tube covered with a layer of vaseline 1 inch in depth to prevent access of air, and at the end of that time it was capable of giving a brilliant light when mixed with luciferase. Luciferin and luciferase *together* in solution are likewise both stable in the absence of oxygen. I have kept such solutions in an evacuated tube or in a hydrogen atmosphere for many months and at the end of that time on admitting air a brilliant light appears.

The oxidative disappearance of luciferin, like other chemical reactions, is greatly accelerated at the higher temperatures. In a previous paper[1] I called attention to the fact that whereas luciferin was not destroyed by a short boiling, 3 or 4 minutes boiling were sufficient to destroy it. This is entirely a matter of the increased rate of oxidation at the boiling point. We can boil a solution of luciferin without harm for 30 minutes provided the boiling is carried out in a stream of hydrogen which has been rendered free of oxygen by passage over a glowing platinum spiral. Acid retards and alkali favors the oxidation of the luciferin (apart from luciferase). A solution of luciferin made slightly acid with HCl may be boiled for 25 minutes in the air

without complete oxidation, whereas a neutral or slightly alkaline solution is quickly oxidized. A solution of luciferin will keep very well in a tall test-tube if the tube is left undisturbed. Diffusion of oxygen into the depths of the tube is very slow. Perhaps the best way to obtain a concentrated solution of luciferin is to filter the hot water extract of *Cypridina* directly into a tall narrow vessel and pass a current of CO_2 through it while cooling. The slight acidity and the anaerobic conditions both prevent oxidation. A little dilute acetic acid may be used in place of carbon dioxide.

Although both luciferin and oxyluciferin will pass collodion or parchment membranes, I have been unable to obtain them in crystalline form, and presume that they also are present in the colloidal state.[3] The properties of the luciferin in the hot water extract and the luciferase in the cold water extract will be considered under the following heads: Action of enzymes, salting out, alcohol and acetone, solubility in organic solvents, alkaloidal reagents, heavy metal salts, acids and alkalies, adsorbents.

Action of Enzymes.

Table I gives the results of enzyme experiments. A solution of crude luciferase (or crude luciferin) was mixed with the enzyme preparation and kept at 38°C. in an incubator for from 18 hours to 4 days. Controls were always employed, using previously boiled enzyme solution. Experiments were also made to determine whether the particular enzyme preparation was active on its substrate, and no experiments were considered in which this was found not to be the case. After the enzyme solution had acted for the proper length of time on the photogenic substances, their light-giving power was tested by adding an equal amount of luciferin (or luciferase) to both control and active tube and comparing the brightness of the light resulting from the active tube with that of the control. In order to prevent oxidation the digests of luciferin were carried out in long test-tubes, full of solution, which were either corked or covered with a

[3] Dubois regards *Pholas* luciferin as a natural albumin and *Pholas* luciferase as an oxidizing enzyme made up of iron associated with a protein. Dubois, R., La vie et la lumière, Paris, 1914.

thick layer of vaseline. This procedure is not necessary in the case of luciferase.

The salivary diastase was fresh filtered saliva, and the yeast invertase a fresh filtered extract of yeast ground with sand. All the other preparations were made by dissolving the commercial enzyme powder in water. The erepsin was a solution of duodenal scrapings dried *in vacuo* and powdered, and the spleen, liver, and kidney substance was a solution of these glands dried quickly *in vacuo* and powdered; all were supplied by the Digestive Ferments Co. of Detroit. The three latter preparations probably contained proteolytic enzymes although they did not digest fibrin under the same conditions with which they were tested with the photogenic substances. The erepsin in neutral solution did not digest fibrin to any extent, or albumen, but tryptophane was produced from Witte's peptone.

Since both acid and alkali injure luciferase and alkali causes a very rapid oxidation of luciferin, some difficulty was experienced in working with pepsin, active only in acid, and with trypsin, most active in alkaline solution. As preparations containing trypsin were found to digest fibrin fairly rapidly in neutral solution, they were made up in water and the experiments show that neutral trypsin solutions will digest luciferase. Presumably such neutral solutions would also digest luciferin if it were capable of digestion, but the results indicate that it is not. Pepsin could only be tested on luciferase with an amount of HCl lower than the optimum, otherwise the HCl alone is sufficient to destroy the luciferase. For this reason the action of pepsin was not so carefully investigated, but the result of the one experiment recorded indicates that a slow digestion of luciferase occurs. Acid is not so destructive to luciferin, and 0.2 per cent HCl plus pepsin was found to possess no digestive power.

It will be noticed from Table I that of all the enzymes tried on luciferase only the proteolytic enzymes have any digestive power. Trypsin, erepsin, and pepsin HCl all have at least some digestive action. The commercial preparations of pancreas (pancreatin) usually contain some lipase (steapsin) and diastase (amylopsin), but as salivary diastase (ptyalin) and malt diastase did not digest luciferase and a sample of trypsin lacking lipase did digest luciferase, the destructive power of various pancreatin and steapsin preparations is

TABLE I.

Enzyme solution.	Action of enzymes on luciferase, at 38°C.			
	Digestive power tested.	Preservative.	Light with	
			24 hrs.	48 hrs.
Malt diastase*.....	Starch digestion.	None.	Brilliant.	
Same boiled.....	No digestion.	"	"	
Salivary diastase.....	Starch digestion.	None.	Brilliant.	
Same boiled.....	No digestion.	"	"	
Yeast invertase.....	Cane sugar digestion.	Toluene.		
Same boiled.....	" " "	"		
Pepsin† in less than 0.2 per cent HCl.....	Coagulated albumen digestion.	Xylene.	Good.	
Same boiled.....	No digestion.	"	Brilliant.	
Trypsin‡.....	Fibrin but not olive oil digestion.	Toluene.	Faint.	Negative.
Same boiled.....	No digestion.	"	Brilliant.	Bright.
Pancreatin§ in 0.5 per cent Na_2CO_3.....	Albumen and starch digestion.	Xylene.	Fair.	Faint.
Same boiled.....	No digestion.	"	Brilliant.	Brilliant.
Pancreatin*.....	Fibrin and starch digestion.	Toluene.	Negative.	
Same boiled.....	No digestion.	"	Bright.	
Steapsin‖.....	Fat, fibrin, and starch digestion.	Toluene.	Faint.	Negative.
Same boiled.....	No digestion.	"	Brilliant.	Bright.
Rennin¶.....	Coagulated milk.	None.	Bright.	
Same boiled.....	No coagulation.	"	"	
Erepsin‖.....	Starch and peptone but not fibrin or albumen digestion.	Toluene.	Fair.	Negative.
Same boiled.....	No digestion.	"	Bright.	Bright.
Arlco urease.....	Urea hydrolyzed.	Toluene.	Bright.	
Same boiled.....	No action.	"	"	
Spleen (calf) substance‖.....	Olive oil but not fibrin digestion.	Toluene.	Good.	Faint.
Same boiled.....	No digestion.	"	Bright.	Bright.
Kidney (beef) substance‖.....	Fibrin or oil not digested.	Toluene.	Good.	Fair.
Same boiled.....	No digestion.	"	"	"
Liver (hog) substance‖.....	Olive oil but not fibrin digestion.	Toluene.	Good.	Faint.
Same boiled.....	No digestion.	"	Bright.	Bright.

* Merck. † Merck, U. S. P. ‡ Fairchild, Bros. and Foster.

TABLE I.

Water (unless stated otherwise).			Action of enzymes on luciferin at 38°C. kept in absence of oxygen.				
Luciferin after		Remarks.	Preservative.	Light with luciferase after			Remarks.
72 hrs.	96 hrs.			18 hrs.	24 hrs.	48 hrs.	
			None.	Good.			
			"	"			
			None.			Good.	Repeated with same result.
			"			"	
Bright.		Repeated twice with same result.	None.		Good.		
"			"		Fair.		
Good.				Fair.			Solution neutralized before testing.
Brilliant.				"			0.2 per cent HCl used.
	Faint.	Luciferase concentrated.	Toluene.			Bright.	Neutral pancreatin used.
	Brilliant.	Repeated twice with neutral pancreatin and same result.	"			"	
			Toluene.			Bright.	
			"			"	
		Note that this solution contains trypsin.	Toluene.			Bright.	Repeated twice; same result.
			"			"	
			None.	Fair.			
			"	"			
		Repeated with same result.	Toluene.			Bright.	Repeated twice; same result.
			"			"	
			None.	Good.			
			"	"			
Very faint.	Negative.		None.		Good.		
Bright.	Bright.		"		"		
Fair.	Fair.		None.		Good.		
"	"		"		"		
Faint.	Negative.		None.		Good.		
Bright.	Bright.		"		"		

‖ Parke Davis Co. ‖ Digestive Ferments Co. ¶ Chr. Hansen.

unquestionably due to their trypsin content. That the destruction of luciferase is actually due to digestion and not to the injurious action of amino-acids resulting from the digestion of proteins associated with the luciferase is shown by adding the products of a 4 day (at 38°C.) tryptic digest of albumen (then boiled to destroy the trypsin) to luciferase, and keeping the mixture with toluene at 38°C. for 4 days more. The luciferase was found to be unaffected by the amino-acids present.

These experiments all indicate that luciferase is a protein. As erepsin has a digestive action one might suppose that it belongs to the group of proteoses, but too great reliance cannot be placed on conclusions drawn from the action of erepsin, as this enzyme is said to hydrolyze histones, protamines, casein, fibrin, and nucleic acid in addition to proteoses and peptones.[4] Dubois finds that *Pholas* luciferase is digested by trypsin.[5]

On the other hand, none of the enzyme preparations tested had any action on luciferin. The proteolytic enzymes especially were studied with great care and with the result that no digestive action could be demonstrated. In one experiment not recorded in Table I luciferin was mixed with a Parke Davis Co. pancreatin preparation, having active proteolytic power and kept at 38° for 4 days without digesting the luciferin. Erepsin also had no digestive action after 4 days at 38°. Merck's pancreatin (without toluene) had no digestive action after 8 days at 38°C. Dried powdered *Cypridinæ* were mixed with Merck's pancreatin and toluene and kept at 38°C. for 8 days. This digest was found to contain no luciferase but abundant luciferin. There is no doubt of the non-digestibility of the latter. As already mentioned, these digests must be carried out in absence of oxygen, otherwise the luciferin undergoes spontaneous oxidation to oxyluciferin and disappears apart from any enzyme action. The oxyluciferin like luciferin itself is also undigested after 4 days action of a pancreatin solution at 38°C. As all common proteins, except the racemized proteins and certain very insoluble albuminoides (elastin

[4] Samuely, F., in Oppenheimer, C., Handb. Biochem. des Menschen und der Tiere, Jena, 1909, i, 554.

[5] Dubois, R., *Ann. Soc. Linn. Lyons*, 1914, xli, 161.

and keratin), are digested by trypsin, these experiments indicate that luciferin is not a common protein, but they give us no idea of the class to which it belongs.

Salting Out.

If crude luciferase solution is saturated with crystals of NaCl at 20°C. no precipitate forms but only a slight turbidity appears. The solution filters turbid and luciferase is found unharmed in the filtrate. Saturation with NaCl is a good method of preserving luciferase from the growth of moulds and bacteria.

One-half saturation with $MgSO_4$ also produces a slight turbidity and luciferase is found unharmed in the filtrate. Complete saturation with $MgSO_4$ produces a fine precipitate, again dissolving in water, which contains considerable luciferase. The opalescent filtrate also contains some luciferase so that saturated $MgSO_4$ precipitates luciferase partially but not completely.

One-half saturation with $(NH_4)_2SO_4$ produces a precipitate which contains very little luciferase after washing with half saturated $(NH_4)_2SO_4$. Most of the luciferase is found in the filtrate. On saturation with $(NH_4)_2SO_4$ an abundant precipitate forms and *no* luciferase remains in the filtrate. The $(NH_4)_2SO_4$ precipitate dissolves completely in water and gives a brilliant light if mixed with luciferin.

If a crude solution of luciferin is saturated with NaCl or half saturated with $MgSO_4$ or half saturated with $(NH_4)_2SO_4$ no precipitate forms. The luciferin remains in solution. With saturated $MgSO_4$ the luciferin is partially precipitated; with saturated $(NH_4)_2SO_4$ it is almost completely precipitated, but a small amount of luciferin still remains in the filtrate. The addition of acetic acid to the point where precipitation of the crude luciferin solution is complete (probably a nucleoprotein or mucin is precipitated) and subsequent careful saturation with $MgSO_4$ or $(NH_4)_2SO_4$ does not completely precipitate the luciferin.

The above results can be confirmed by adding saturated solutions of NaCl or $MgSO_4$ or $(NH_4)_2SO_4$ to dry powdered *Cypridinæ*. The powder glows strongly in saturated NaCl which dissolves both lucif-

TABLE II.

Properties of Photogenic Substances.

Property.		Luciferase.	Luciferin.
Salting out by	Saturation NaCl	Not precipitated.	Not precipitated.
	Half saturation $MgSO_4$	" "	" "
	Saturation $MgSO_4$	Nearly completely precipitated.	Partially precipitated.
	" " + acetic acid		" "
	Half saturation $(NH_4)_2SO_4$	Slightly precipitated.	Not precipitated.
	Saturation $(NH_4)_2SO_4$	Completely precipitated.	Nearly completely precipitated.
	" " + acetic acid		" " "
Solubility in	Methyl alcohol	Insoluble.	Soluble.
	Ethyl "	"	"
	" " 90 per cent	"	"
	" " 70 " "	"	"
	" " 50 " "	Slightly soluble.	"
	Propyl "	Insoluble.	"
	Isobutyl "	"	Fairly soluble.
	Amyl "	"	Slightly "
	Benzyl "	"	Soluble.
	Acetone	"	Fairly soluble.
	" 90 per cent	"	Soluble.
	" 70 " "	Slightly soluble.	"
	" 50 " "	Fairly soluble.	"
	Ethyl acetate	Insoluble.	"
	" propionate	"	Fairly soluble.
	" butyrate	"	" "
	" valerate	"	Slightly "
	" nitrate	"	Very slightly soluble.
	Glycerol	"	Soluble.
	Glycol	"	"
	Ether	"	Insoluble.
	Chloroform	"	"
	Carbon disulfide	"	"
	" tetrachloride	"	"
	Benzene	"	"
	Toluene	"	"
	Xylene	"	"
	Petroleum ether	"	"
	Aniline	"	"
	Glacial acetic acid	"	Fairly soluble.

erin and luciferase; weakly, in saturated $MgSO_4$ which dissolves some luciferin but very little luciferase; and not at all in saturated $(NH_4)_2SO_4$ which dissolves no luciferase. On filtering the saturated $(NH_4)_2SO_4$ extract of dry *Cypridinæ* and pouring this into water, no light appears, but if luciferase is now added, a faint light appears, showing that a small amount of luciferin has gone into solution. These results are summarized in Table II.

Although we ordinarily think of the proteins as the substances, ***par excellence***, capable of being salted out of solution, the property is characteristic of many emulsion colloids, notably soaps, polysaccharides, and phospholipins. However, neither luciferase nor luciferin is a soap because they are not precipitated by $CaCl_2$; nor phospho- or galactolipins, because they are both insoluble in ether and benzene, hot or cold. It is possible that they are of polysaccharide nature as starch and glycogen are nearly if not completely precipitated by saturating their solution with $(NH_4)_2SO_4$. But the polysaccharides are not precipitated by phosphotungstic acid, whereas luciferase is completely precipitated and luciferin very nearly completely precipitated (see p. 283).

The evidence from salting out experiments indicates that both luciferin and luciferase are proteins, the former on the border-line between proteoses and peptones, the latter a more complicated protein but not a globulin. Dubois[5] finds *Pholas* luciferin completely precipitated by saturation with $(NH_4)_2SO_4$ but not by $MgSO_4$ or NaCl.

Alcohol and Acetone.

If strong ethyl alcohol or acetone is added to a solution of crude luciferase, an abundant precipitate forms. This precipitate is found to contain the luciferase which is separated completely from solution by alcohol between 50 and 60 per cent, and by acetone between 70 and 80 per cent. We are dealing in both cases with a precipitation and not a coagulation of luciferase. The precipitates partially redissolve in water and if the solution is filtered luciferase is found in the filtrate. Indeed, the precipitates from addition of alcohol or acetone to crude luciferase may stand under alcohol (95 per cent) or acetone (90 per cent) respectively for 35 days without complete de-

struction of the luciferase, but there is always a diminution of activity which is no doubt eventually complete. The alcohol or acetone precipitate may be washed and dried and will give light whenever luciferin is added.

If strong ethyl alcohol or acetone is added to a solution of crude luciferin, a precipitate also forms, but it is not nearly so voluminous, since the heat-coagulable proteins are absent from the luciferin solution. With alcohol the precipitate is very fine; with acetone it is flocculent and clumps together readily. The precipitates washed once with 95 per cent alcohol and acetone respectively dissolve completely in water but these solutions give only a faint or no light if luciferase is added. On the other hand, the filtered alcohol and acetone solutions contain considerable amounts of luciferin. The absence of luciferin in the alcohol and acetone precipitates is only apparent. It is really partly precipitated by alcohol and acetone but is largely oxidized during the process of testing. Experiments indicate that oxyluciferin is present in the precipitates and can be reconverted into luciferin by appropriate methods. These experiments indicate that alcohol and acetone only partially precipitate luciferin, but completely precipitate luciferase. Boiling absolute alcohol will extract a considerable amount of luciferin and such a solution is quite stable even if exposed to air provided the alcohol is not allowed to evaporate. In alcohol-water and acetone-water mixtures the luciferin oxidizes readily and disappears in the course of a day or so.

Solubility in Organic Solvents.

The solubility of luciferin and luciferase in a number of pure organic solvents was tested by extracting the dried powdered *Cypridinæ*, filtering, and then testing the filtrate by the addition of luciferase and luciferin, respectively. Small pieces of the photogenic gland will sometimes pass through ordinary filter paper, but can always be recognized as isolated brightly luminous dots appearing when the solvent is tested for photogenic material. A heavy blotting paper holds back these fine particles and was used for filtering except in the case of very viscous solvents. The results are summarized in Table II.

It will be noticed that luciferase is not extracted by any of the solvents tried, whereas luciferin is soluble in a considerable number of

them. Methyl alcohol, ethyl alcohol, propyl alcohol, benzyl alcohol, ethyl acetate, glycerol, and glycol all dissolve a considerable amount of luciferin. Solvents non-miscible with water, such as benzyl alcohol or ethyl acetate, give up their content of luciferin to the water phase and it then luminesces if luciferase is present. In the case of the homologous series of aliphatic alcohols, the higher the alcohol in the series the less luciferin will it dissolve. The same is true for the homologous series of esters.

Luciferin is fairly stable in methyl alcohol, ethyl alcohol, propyl alcohol, benzyl alcohol, and glycerol if no water is present, but rather quickly disappears in acetone, glycol, and ethyl acetate, presumably because oxidation occurs more rapidly in the latter solvents. In the typical fat solvents as ether, chloroform, benzene, etc., the luciferin is insoluble. Luciferin is also soluble in glacial acetic acid but not in aniline.

There is nothing in the solubility relations of luciferase to indicate that it is not a protein. On the other hand, the solubilities of luciferin are certainly unusual for a natural protein. The best known class of proteins soluble in alcohol is the protamines of plants, but the protamines are insoluble in water and absolute alcohol. Zein, the protamine of corn, is soluble in 90 per cent ethyl alcohol, methyl and propyl alcohols, glycerol heated to 150°C., and glacial acetic acid.[6] Recently Osborne and Wakeman[7] have described a protein from milk having solubilities similar to those of gliadin, the protamine of wheat. Welker[8] has described a substance, obtained from Witte's peptone giving the biuret, Millon, and Hopkins-Cole tests, which is soluble in water and absolute alcohol but not in ether, and it is possible that others of the peptones are soluble in absolute alcohol. On the other hand, some proteins in the absence of salts form colloidal solutions in strong alcohol from which they may be precipitated by an appropriate salt.[9] As the absolute alcohol extract of *Cypridinæ* was made from dry material containing the salts of sea water, some salt was present, but there is always the possibility of sol formation.

[6] Osborne, T. B., *Ergebn. Physiol.*, 1910, x, 47.
[7] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1918, xxxiii, 243.
[8] Welker, W. H., *Biochem. Bull.*, 1912–13, ii, 70.
[9] Private communication from T. B. Osborne.

If we extract dried *Cypridinæ*, which have previously been thoroughly extracted with benzene, with 800 cc. of boiling absolute alcohol for an hour, filter the alcohol extract through blotting paper and hardened filter paper, quickly evaporate the filtrate to dryness on the water bath, and dissolve the residue in a small quantity of water saturated with CO_2, we obtain a yellow opalescent solution which gives a bright light with luciferase. This solution contains some protein or protein derivatives as it gives a very faint Millon reaction, a good positive ninhydrin test, reddish blue in color, but no biuret reaction. It precipitates with tannic and phosphotungstic acids but not with picric, acetic, trichloroacetic, or chromic acids or on saturation with $(NH_4)_2SO_4$. The phosphotungstic precipitate is not due to the presence of calcium. The extract gives a faint Molisch reaction for carbohydrates. As the evidence points to the presence of some protein material in the absolute alcohol extract of *Cypridinæ*, it is possible that this protein is luciferin. It should be emphasized, however, that the Millon reaction was very faint although the ninhydrin was quite marked.

Alkaloidal Reagents.

A solution of phosphotungstic acid added to crude luciferase solution gives a voluminous precipitate which, if washed quickly in running water on the filter and then suspended in water, does not completely dissolve but gives a good light if luciferin is added. It dissolves if a trace of dilute NH_4OH solution is added. The filtrate gives no light with excess luciferin, even though it contains some material which will precipitate on addition of more phosphotungstic acid.

Tannic acid added in slight excess to luciferase gives an abundant precipitate which contains luciferase and will give a good light with luciferin if suspended in water. It does not completely dissolve, even on addition of dilute NH_4OH solution. The filtrate gives a very faint light on addition of an excess of luciferin, even though an excess of tannic acid was used to precipitate and all precipitable material was thrown down.

Saturated aqueous picric acid added in equal volume to crude luciferase solution gives a not very abundant precipitate which if

filtered off, washed quickly with water, and suspended in water, does not completely dissolve. If luciferin is added the precipitate does dissolve and a good light appears. Dilute NH_4OH solution causes the precipitate to dissolve. The filtrate gives a very faint light when an excess of luciferin is added, even though everything was precipitated that would precipitate with picric acid and an excess of the latter was present. The excess of picric acid was not sufficient to harm the luciferin used in testing.

Luciferase is, therefore, completely precipitated by phosphotungstic acid and almost completely precipitated by tannic and picric acids without harm.

Phosphotungstic acid added to a solution of crude luciferin to slight excess gives a bulky precipitate which can be filtered off and the filtrate is perfectly clear. It gives no further precipitate with phosphotungstic acid but contains a small amount of luciferin, as a faint light appears when luciferase in *excess* (since luciferase is also precipitated by the excess phosphotungstic acid present) is added. The precipitate washed on the filter with dilute phosphotungstic acid does not dissolve completely in water but partly dissolves forming an opalescent solution. It is brought into solution by adding crude luciferase or a trace of dilute NH_4OH solution. The precipitate contains luciferin and gives a brilliant light with luciferase. It is probably the slight alkalinity of the luciferase solution which causes resolution of the luciferin precipitate. The addition of a small amount of HCl to the phosphotungstic-luciferin filtrate causes further precipitation, leaving a clear supernatant fluid containing no trace of luciferin.

Tannic acid likewise gives a voluminous precipitate with luciferin solutions and the filtrate is clear at first but may become cloudy in a short time, especially at the surface, unless considerable tannic acid is added. If an excess of tannic acid is present, as indicated by absence of further precipitate on adding more tannic acid, a small amount of luciferin is present in the filtrate but practically all is precipitated. The precipitate does not completely dissolve in water even if NH_4OH is added, but gives a bright light when luciferase is added. The addition of a small amount of acetic acid + NaCl or HCl does not cause further precipitation. Both the phosphotungstic

and tannic acid precipitates can be dried and will again give light with luciferase if redissolved.

TABLE III.

Properties of Photogenic Substances.

Property.		Luciferase.	Luciferin.
Alkaloidal reagents	Phosphotungstic acid	Completely precipitated.	Very nearly completely precipitated.
	" and acetic acids		" " "
	" acid and HCl		Completely precipitated.
	Tannic acid	Nearly completely precipitated.	Nearly completely precipitated.
	" and acetic acids		" " "
	" acid and HCl		" " "
	Picric "	Nearly completely precipitated.	Not precipitated.
	" and acetic acids		" "
	" acid and HCl		" "
	$K_4Fe(CN)_6$ and acetic acid		" "
Heavy metal salts	Basic lead acetate	Completely precipitated.	Not completely precipitated.
	Neutral lead acetate	Nearly completely precipitated.	" " "
	" " " and acetic acid		Not precipitated.
	Mercuric chloride	Not precipitated.	Not completely precipitated.
	" " and acetic acid		Almost completely precipitated.
	Uranyl nitrate and acetic acid		Not completely precipitated.
Acids and alkalies	NaOH	Not precipitated.	Not precipitated.
	NH_4OH	" "	" "
	Acetic acid	" "	" "
	H_2CO_3	" "	" "
	Trichloroacetic acid	" "	" "

Picric acid added to luciferin solution gives no good precipitate but only an opalescence or turbidity. This is true even when picric acid

is present in considerable excess, as on addition of an equal volume of saturated aqueous picric acid. Further addition of a little dilute acetic acid or HCl does not cause precipitation. The solution filters turbid and luciferin may be demonstrated in the filtrate on adding luciferase. A yellow light due to the picric acid in solution appears. A little dilute NH_4OH clears the turbidity.

If we add acetic acid to luciferin solution until the precipitation (probably a precipitate of mucin and nucleoprotein) is complete and then dilute $K_4Fe(CN)_6$ solution, no further precipitate forms and luciferin is found abundantly in the filtrate which gives no further precipitate with $K_4Fe(CN)_6$ and acetic acid. These results are recorded in Table III.

Luciferin is, therefore, very nearly completely precipitated by phosphotungstic and tannic acids but not by picric acid. It is completely precipitated unharmed by phosphotungstic acid + HCl, but not by tannic acid + HCl or picric acid + HCl. It is not precipitated by potassium ferrocyanide and acetic acid. Dubois[10] finds *Pholas* luciferin to be completely precipitated by picric acid.

Heavy Metal Salts.

Some of the heavy metal salts (Pb acetate, $AgNO_3$, $CuSO_4$) precipitate in sea water. As both crude luciferin and luciferase solutions, although prepared with distilled water, contain small amounts of the salts of sea water, a slight precipitate would be formed upon addition of lead acetate. It is too small in amount to interfere with the action of lead acetate as a precipitant, but it must be borne in mind that some of the luciferin or luciferase might be adsorbed on any precipitate formed, as this is especially likely to occur with inorganic precipitates. Another difficulty encountered in precipitating protein solutions with heavy metal salts is that in excess of heavy metal salt some of the precipitate may redissolve. However, certain definite results were obtained by precipitating crude luciferin and luciferase with lead and mercury salts (the usual protein precipitants) and these are described below although their interpretation may be open to question.

[10] Dubois, *Ann. Soc. Linn. Lyons*, 1913, lx.

Neutral lead acetate solution was added to crude luciferase solution, drop by drop, until no further precipitate formed. The filtrate was clear and gave no further precipitate on adding more lead acetate. It gave a good light with excess of luciferin. The precipitate suspended in water does not dissolve, but upon addition of luciferin gives a bright light.

Basic lead acetate added drop by drop gives a bulky precipitate leaving a clear filtrate which gives no further precipitate on addition of more basic lead acetate. This filtrate gives no light with excess luciferin but the precipitate washed once with water on the filter gives a bright light.

Saturated $HgCl_2$ solution was added drop by drop to luciferase solution until no further precipitate occurs. The filtrate is perfectly clear and gives a bright light with luciferin although it gives no further precipitate with $HgCl_2$. The precipitate washed on the filter for 20 hours in running water is suspended in water. It does not dissolve but on addition of luciferin gives a fair light.

Luciferase therefore is completely precipitated by basic lead acetate, nearly completely precipitated by neutral lead acetate, but not precipitated by mercuric chloride.

If a saturated solution of $HgCl_2$ is added drop by drop to luciferin solution a precipitate forms. If this is filtered off, washed with dilute $HgCl_2$, and suspended in water, it does not completely dissolve but gives a good light if an excess of luciferase is added. The opalescent filtrate which gives no further precipitate with $HgCl_2$ gave a fair light on adding an excess of luciferase. Mercuric chloride and acetic acid almost completely precipitate luciferin from solution.

Neutral lead acetate solution added to luciferin gives a heavy precipitate. The solution filters cloudy at first even though no further precipitate forms on adding lead acetate, but soon filters clear. This clear filtrate containing some Pb gives a bright light on addition of excess of luciferase. The precipitate was washed several times on the filter and by decantation with dilute Pb acetate, and suspended in water. It does not completely dissolve but gives a good light with luciferase. Lead acetate and acetic acid give no good precipitate with crude luciferin.

Basic lead acetate gives a voluminous heavy precipitate with crude luciferin. After heating, the solution was filtered and the clear filtrate, which gave no further precipitate with basic lead acetate, gave a brilliant light with luciferase. The precipitate washed twice with water on the filter does not dissolve in water, but suspended in water gives a faint light on adding luciferase. Uranium nitrate + acetic acid does not completely precipitate luciferin from solution. These results are recorded in Table III.

Luciferin is therefore not completely precipitated from solution by mercuric chloride with or without acetic acid, neutral lead acetate or basic lead acetate, or uranium nitrate and acetic acid.

Acids and Alkalies.

As alkalies (KOH or NaOH in small, NH_4OH in greater concentration) precipitate the Mg of sea water, this salt should be removed from a crude solution of luciferin and luciferase before studying the precipitating effects of the substances. This can be done by the addition of a small amount of sodium pyrophosphate which forms Ca and Mg pyrophosphates insoluble in water. The precipitates are removed by filtration and the photogenic substances are found unharmed by the addition of pyrophosphate.

Neither luciferin nor luciferase is precipitated by addition of dilute NaOH or dilute NH_4OH to their crude solutions, first rendered free of Ca and Mg.

Dilute acetic acid added to luciferase solution gives a fine precipitate which is filtered off and washed with running water for 24 hours. It does not dissolve completely in water and gives only a faint light with luciferin. The precipitate is probably a mucin and carries down some adsorbed luciferase. The filtrate is perfectly clear, gives no further precipitate (sometimes a slight cloudiness on standing) with acetic acid, but a bright light with luciferin. The addition of slightly more acetic acid results in a clear filtrate giving no light with excess luciferin. The precipitate on the filter does give a faint light with excess luciferin but appears to be injured by the acid.

Saturation with CO_2 causes a precipitation in crude luciferase solution, but the filtrate gives a brilliant light with luciferin.

Dilute acetic acid added to concentrated luciferin gives a stringy precipitate. If filtered off, the filtrate is slightly opalescent but does not become more cloudy or precipitate if more acetic acid is added. It gives a bright light if luciferase is added to it. The precipitate does not completely dissolve in water, but if washed with water and suspended in water gives a fair light with luciferase. This precipitate is probably a mucin containing some adsorbed luciferin. In the luminous gland of *Cypridina* there is a material which stains as does mucin and it would be found in the hot water extract of the whole animal. The acetic acid precipitate does not form readily if the luciferin solution is hot when acetic acid is added. A little dilute NH_4OH will dissolve the precipitate and it can be reprecipitated by acetic acid. The filtrate from the acetic acid precipitation gives a voluminous precipitate with phosphotungstic acid which does not carry down all the luciferin unless a little HCl is also added, when luciferin is completely precipitated and may be demonstrated in the precipitate.

Dilute HCl alone gives a precipitate with crude luciferin solution but it almost completely dissolves in an excess of dilute HCl.

Saturation of a solution of crude luciferin in presence of some NaCl with carbon dioxide does not cause precipitation or an increase in turbidity.

Dilute trichloroacetic acid gives a stringy precipitate with crude luciferin solution similar to that with acetic acid, but abundant luciferin is found in the clear filtrate which gives no further precipitate with trichloroacetic acid. These results are recorded in Table III.

Hence neither luciferin nor luciferase is precipitated from crude solution by dilute NH_4OH or NaOH or by dilute acetic acid, and neither of them can belong to the group of histones (precipitated by dilute NH_4OH) or mucins, or nucleoproteins, or such phosphoproteins as caseinogen, which are precipitated by dilute acetic acid. A mucin or nucleoprotein is precipitated from both crude luciferase and luciferin solution by dilute acid and carries down some luciferase and some luciferin in the adsorbed state. It is easy to demonstrate that such an adsorption might occur. A solution of sodium caseinogenate mixed with either luciferin or luciferase and precipitated with acetic acid will carry down a considerable amount, but not all of the luciferin

or luciferase. Luciferin is not, but luciferase is injured by an excess of dilute acetic acid.

Dubois[5] reports that *Pholas* luciferin is not precipitated by carbonic acid in neutal solutions or by acetic acid except in presence of neutral salts and that it forms an insoluble alkali albuminate with NH_4OH. The latter is possibly a $Mg(OH)_2$ formed from the magnesium in the luciferin solution.

Adsorbents.

Proteins are usually separated from their solutions by one or another of the following methods: heat coagulation (in trace of acid); precipitation by alcohol or acetone (in large excess); precipitation by heavy metal salts (basic lead acetate, $HgCl_2$ and acid, uranium acetate and acid, etc.); alkaloidal reagents (phosphotungstic, tannic, picric acids, etc.); salting out (by $MgSO_4$ and acid, $(NH_4)_2SO_4$, etc.); digestion by proteolytic enzymes; adsorption (by chloroform, toluene, $Fe(OH)_3$, kaolin, and gum mastic).

We have already noted the behavior of luciferase and luciferin toward the first six methods. Both of these substances can also be separated from solution by adsorption on appropriate material; in fact, they are rather readily adsorbed especially by inorganic precipitates. Their complete adsorption is usually merely a matter of obtaining sufficient adsorbing surface area. For this reason comparative studies on adsorption of different materials are difficult to carry out because we cannot be sure of uniform surface area. However, it may be of interest to record a few of the experiments on adsorption.

A neutral dilute solution of luciferase was found to be completely adsorbed by bone-black, $Fe(OH)_3$, As_2S_3, infusorial earth (SiO_2), talc, and kaolin; nearly completely adsorbed by asbestos, pumice, $CaCO_3$, and $MgCO_3$; not nearly completely adsorbed by ground glass, sulfur powder, gelatin or agar-agar threads, heat-coagulated egg albumin, fresh precipitated caseinogen, cotton, or cornstarch.

A solution of luciferase shaken with five successive additions of fresh chloroform, until the chloroform is no longer emulsified but separates as readily as with water, is reduced considerably in luciferase content but the luciferase is not completely removed by the chloroform.

Neutral luciferin is completely adsorbed by bone-black, $Fe(OH)_3$, kaolin, talc, and $CaCO_3$, but not by many organic precipitates as caseinogen, cornstarch, or gelatin threads. There is the difficulty in studying adsorption of luciferin that oxidation may be accelerated by the presence of finely divided material.

Luciferin can also be removed practically completely from solution by gum mastic according to the method of Michaelis and Rona[11] for removing proteins from blood serum.

CONCLUSIONS.

There seems to be very little doubt but that luciferase is a protein or so closely associated with proteins that their removal destroys its characteristic properties. The particular group of proteins to which it belongs may be arrived at by a process of exclusion, and only the group of albumins has properties which agree completely with those of luciferase.

Dubois believes *Pholas* luciferase to be an oxidizing enzyme similar to the oxydones of Battelli and Stern[12] because it is readily destroyed by fat solvents such as chloroform, strong alcohol, etc. He has detected iron in a luciferase solution which has dialyzed against running water for a long time, and believes it to be made up of protein in combination with iron and to act as an "oxyzymase ferrique."[5] *Cypridina* luciferase, on the other hand, is not *readily* destroyed by fat solvents. Toluene and chloroform are good preservatives, and I often make use of them for this purpose, keeping the luciferase solutions for many months. Professor A. H. Phillips of Princeton University has very kindly analyzed some whole dried *Cypridinæ* for me and finds iron, copper, and manganese but no zinc or vanadium to be present. Whether these metals are connected with the action of *Cypridina* luciferase is uncertain, but it is significant that all three of the metals thought to be concerned in organic oxidations are present.

Although a large amount of luciferin mixed with a small amount of

[11] Michaelis, L., and Rona, P., *Biochem. Z.*, 1907, ii, 219; Rona and Michaelis, *ibid.*, 1907, iii, 109; Michaelis and Rona, *ibid.*, 1907, iv, 11.

[12] Battelli, F., and Stern, L., *Biochem. Z.*, 1914, lxvii, 443.

luciferase will use up all the latter,[13] I agree with Dubois that luciferase has sufficient properties in common with the enzymes as a class to be considered an enzyme. The peroxidases are well known to be used up in the reactions they accelerate. All workers on enzymes agree that the more enzymes are purified the less active they become. The chemical procedures necessary to remove foreign material bring about irreversible changes in the enzyme itself, a characteristic also of many protein groups and of the colloidal state in general. This is true in the case of luciferase, for the crude luciferase solution is the most active preparation that can be obtained.

I believe that *Cypridina* luciferase should be placed in a class of oxidizing enzymes by itself—a group having the chemical reactions of an albumin, possibly in combination with some heavy metal, and which as far as we know, acts specifically on only one substance, *Cypridina* luciferin. It resembles the plant peroxidases in resisting the action of chloroform, toluene, etc., but will not oxidize any of the hydroxyphenol or aminophenol compounds[14] so readily oxidized by the

[13] If concentrated luciferin and weak luciferase are mixed, the light which appears will last a long time before going out. After the light disappears, if this mixture is diluted with water or more luciferin is added, no further luminescence occurs, but if more weak luciferase is added, light again is produced and lasts a considerable time. The fact that no more light appears on diluting the concentrated luciferin-weak luciferase mixture with water shows that the enzyme has not been inhibited by reaction products. If so, the dilution of these reaction products should allow the system to proceed to a new (false) equilibrium with production of light. Dubois (*Ann. Soc. Linn. Lyons*, 1917, lxiv, 105) has misunderstood my previous statement regarding this.

[14] Because of the ease with which many of these hydroxyphenyl compounds undergo autooxidation, one must always compare the color produced by luciferase solution with that produced in a control of boiled luciferase solution. I find that a concentrated luciferase solution well shaken with chloroform and filtered, which produced a brilliant light with luciferin, had no oxidative action on phenol, α-naphthol, *p*-phenylenediamine, ortol, orcinol, hydrochinon, resorcinol, pyramidon, phloroglucin, pyrocatechol, gallic acid, benzidine, pyrogallol, gum guaiac, amidol, tannin, or α-naphthylamine, either with or without H_2O_2. Dubois (*Ann. Soc. Linn. Lyons*, 1914, xli, 161) reports oxidation of pyrogallol, tannin, hydroquinone, guaiacol, Tromsdrof reagent, chlorohydrate of diaminophenol, *p*-phenylenediamine, and naphthol, naphthol B, and gum guaiac plus H_2O_2 by a solution of *Pholas* luciferase. These results are of little value, however, as there is no evidence that the oxidation is due to luciferase rather than the oxidizing enzymes which one finds in cell extracts of all animals whether luminous or non-luminous.

peroxidases, nor will the peroxidases oxidize luciferin with light production. Dubois' researches show that *Pholas* luciferase differs in some properties from *Cypridina* luciferase, and my own work[15] indicates that firefly luciferase is more like that of *Pholas*. A comparative study of other species of luminous animals is needed in order to delimit more accurately the class of luciferases as a whole.

Luciferin presents many characteristics in common with the proteins, but two, which, to say the least, throw doubt on its protein nature: (1) its peculiar solubility (in alcohols, esters, and glacial acetic acid), (2) and its resistance to digestion by proteases, even by trypsin which has almost universal digestive action. These two peculiarities have been discussed above. We can only say that if a protein, luciferin must belong to a new group differing from *known natural* proteins in these respects. In general characteristics this new group would fall somewhere on the border-line between the proteoses and peptones. It would not be surprising to find in nature proteoses or peptones soluble in absolute alcohol. We know also that some NH-CO linkages of proteins are broken down with great difficulty by trypsin as it is difficult to obtain a tryptic digest of protein which does not give the biuret reaction, and the work of Fischer and Abderhalden has shown that certain artificial polypeptides are not digested by pure activated pancreatic juice. We have, then, three possibilities. Luciferin is (1) either a natural proteose not attacked by trypsin, or (2) if attacked by trypsin, its decomposition products (presumably amino-acids) still contain the group oxidizable with light production, or (3) it is not a protein at all. I believe that luciferin has too many protein characteristics to conform to the last possibility. I have been unable to oxidize with light production various mixtures of amino-acids (from beef and casein) by means of luciferase and consequently am led to believe that luciferin is a new natural proteose, soluble in absolute alcohol and not digested by trypsin.

Dubois believes *Pholas* luciferin to be a natural albumin with acid properties. *Cypridina* luciferin could not possibly be regarded as an albumin, but it is very likely that the luciferins of different species of luminous animals differ in certain characteristics. As in the case of

[15] Harvey, *Am. J. Physiol.*, 1917, xlii, 342.

the luciferases, we know that the luciferins are not identical substances, and only future work can determine in what particulars they differ.

A summary of the properties of *Cypridina* luciferin and *Cypridina* luciferase will be found in the tables accompanying this paper.

THE TEMPERATURE COEFFICIENT OF PHOTOSYNTHESIS.

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The temperature coefficient of photosynthesis presents an interesting problem. On the one hand is the fact that light reactions have very low temperature coefficients; while on the other hand it appears that the temperature coefficients of photosynthesis reported by various investigators[1] are as high as those of ordinary reactions. It should be noted, however, that in a recent investigation van Amstel[2] found a temperature coefficient of 1.26, but expresses doubt whether this really represents the correct value for photosynthesis.

The difficulties of determining temperature coefficients are great in the case of land plants, for when leaves are exposed to sunlight their temperature rapidly rises and it may fluctuate as much as 10°C. in a half hour period. For this reason the writers have employed aquatic plants which form thin layers, the temperature of which can be sufficiently controlled.

In the present investigation the plants were placed in tubes filled with solution; these were immersed in a water bath the temperature of which was kept constant within 1°C., which sufficed for the purposes of the investigation. Thermometers placed in the tubes showed that the temperature did not rise more than 1°C. above that of the bath and that it did not fluctuate more than that of the bath itself. The plant employed in these investigations forms such thin layers

[1] Cf. Matthaei, G. L. C., *Phil. Tr. Roy. Soc., B*, 1905, cxcvii, 47, and the interpretation by Kanitz, A., in Temperatur und Lebensvorgänge, 1915, p. 18. Also Blackman, F. F., and Matthaei, G. L. C., *ibid.*, 1905, lxxvi, 402. Blackman, F. F., and Smith, A. M., *ibid.*, 1911, lxxxiii, 401.

The results of these observers have been interpreted by Brown and Heise as showing very low temperature coefficients (Brown, W. H., and Heise, G. W., *Philippine J. Sc., C.*, 1917, xii, 1).

[2] van Amstel, J. E., *Rec. trav. bot. néerlandais*, 1917, xiii, 1.

(0.078 mm. thick) that its temperature quickly reaches that of the surrounding liquid. This plant is the marine alga *Ulva rigida* (sea lettuce) which was chosen because of its rapid rate of photosynthesis, its hardiness under laboratory conditions, and its excellent mechanical properties.

The method[3] (which seems to be more accurate than those hitherto employed) consisted in placing the plant in sea water to which an indicator had been added, the whole being contained in a tube which was immersed to a depth of 2 or 3 inches in a water bath and exposed to direct sunlight.[4] Photosynthesis was allowed to proceed until a definite amount of CO_2 had been abstracted from the sea water, as shown by the color of the indicator. This was repeated as often as necessary. It has been shown in a previous article[3] that the rate of photosynthesis is not constant from the start, but steadily increases until it reaches a constant value. It was therefore necessary to continue the exposure until the constant value was reached. After determining the constant rate the temperature was changed and new determinations were made.

Since the amount of CO_2 abstracted by the plant (and consequently the amount of photosynthesis) was always the same, the rates at different temperatures are inversely proportional to the time required for the standard amount of photosynthesis.

Since the object of the investigation was to ascertain whether the temperature coefficients are those of light reactions or of ordinary chemical processes, it was not necessary to extend the experiments over a wide range of temperature. The temperatures chosen were 17° and 27°C., which are high enough to ensure rapid photosynthesis but which do not produce the slightest injury.

The results are shown in Table I. For convenience the rate of photosynthesis at 17°C. is taken as 100 and other rates are expressed as per cent of this. Thus the rate at 27°C. is found to be 169 ± 1.[5]

[3] The details of technique are fully explained in a previous article. See Osterhout, W. J. V., and Haas, A. R. C., *J. Gen. Physiol.*, 1918, i, 1.

[4] The tubes were inclined so as to receive the sunlight as nearly as possible at right angles.

[5] This figure represents the average of eight experiments. The results were first expressed as per cent and then averaged.

This is designated in the table as the apparent rate of photosynthesis since it is not corrected for respiration. The correction is made by adding the rate of respiration[6] to the apparent rate of photosynthesis. The sum gives the true rate of photosynthesis.[7] The true rate is seen to be 150 at 17°C. and 271 at 27°C., giving a temperature coefficient of 1.81.[8]

The question arises: How can the process have so high a temperature coefficient? The answer is not far to seek. The writers have suggested in a recent paper[9] that photosynthesis involves catenary reactions of the type $S \rightarrow M \rightarrow P$ in which S represents a substance which, under the influence of light, breaks down to form M;

TABLE I.

Temperature Coefficient of Photosynthesis and Respiration in Ulva.

	Temperature.		Temperature coefficient.
	17°C.	27°C.	
Apparent rate of photosynthesis	100	169±1.1	1.69
Rate of respiration	50±1	102±1.5	2.04
True rate of photosynthesis	150	271	1.81

Each figure represents the average of eight experiments. The rate of photosynthesis at 17°C. (which is taken as 100) represents a change from pH 8.1 to pH 8.3 in 18.1 minutes.

this in turn forms P, the amount of which is proportional to the amount of photosynthesis. If the reaction $S \rightarrow M$ is more rapid than $M \rightarrow P$-

[6] This was ascertained by repeating the experiment with the same pieces of *Ulva* under precisely the same conditions except that light was excluded. It is expressed as per cent of the rate of photosynthesis at 17°C. (which is taken as 100).

[7] This involves the assumption that the rate of respiration is practically the same in light and in dark (as long as the temperature remains the same). There seem to be good grounds for this assumption.

[8] It is not likely that this result is affected by "limiting factors," since the light was practically full sunlight, and since the temperatures were near the optimum and the supply of CO_2 was sufficient, owing to the presence of carbonates and bicarbonates in the sea water. The effect of a limiting factor would be to make the coefficient appear less than it is in reality.

[9] Osterhout and Haas, *J. Gen. Physiol.*, 1918, i, 1.

(as is presumably the case), the speed of the process as a whole will depend chiefly on the speed of $M \rightarrow$ P (the velocity of the whole catenary process being regulated by the speed of the slowest member). It is therefore evident that when the temperature is raised its effect on the process as a whole will depend chiefly on its effect upon $M \rightarrow P$ rather than upon its effect upon $S \rightarrow M$. Hence if the light reaction $S \rightarrow M$ has a low temperature coefficient, while $M \rightarrow P$ (which is not a light reaction) has a high coefficient, the temperature coefficient of the process as a whole will be high. It is therefore not surprising to find that photosynthesis has a temperature coefficient of 1.81. Analogous cases exist in photochemistry.[10]

It is to be expected that similar relations will be found in heliotropic and heliotactic reactions where the stimulus is given by light and the growth or movement which follows is due to a slower process with a coefficient presumably higher than that of the light reaction.[11]

SUMMARY.

The temperature coefficient of photosynthesis in *Ulva* (between 17° and 27°C.) is 1.81. This may be explained by assuming that the process involves a light reaction with a low coefficient followed by an ordinary reaction with a high coefficient.

[10] Cf. Bovie, W. T., *Science*, 1913, xxxvii, 373.

[11] According to T. Nybergh (*Ber. bot. Ges.*, 1912, xxx, 542) this is the case for the oat seedling, but this is disputed by Marie de Vries (*ibid.*, 1913, xxxi, 233; *Versl. wis. en natuurk. Akad. Wetensch. te Amsterdam*, 1913, xxi, 1056.) In certain cases the whole process may depend on the diffusion of a substance (produced in the light) from the point of origin to another region. The coefficient may then be that of diffusion (usually not over 1.28).

A COMPARATIVE STUDY OF PERMEABILITY IN PLANTS.

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The writer has arrived at certain general conclusions concerning the behavior of protoplasm, which are based largely on the study of a single plant. To ascertain whether they have general validity it is necessary to study other plants as well as animals. The following paper gives a brief résumé of results obtained with a red alga (*Rhodymenia palmata*), a green alga (*Ulva rigida*), and a flowering plant (*Zostera marina*). These forms were chosen because they differ greatly from the brown alga *Laminaria Agardhii* (which furnished the material for the experiments hitherto described), not only in the chemical composition of the cell wall[1] but also in the structure of their tissues.

The experiments on *Laminaria* were made by cutting disks from the fronds and packing them in a cylinder, the electrical resistance of which was determined as previously described.[2]

The experiments with *Rhodymenia palmata* (the common dulse of the Atlantic coast) were carried out in precisely the same manner as those on *Laminaria*. This was possible because *Rhodymenia* forms thin sheets of tissue, large enough to be cut by a cork borer into disks 13 mm. in diameter. The disks were packed together, like a roll of coins, to form a cylinder, the electrical conductivity of which was measured as previously described.[2] *Rhodymenia* is much more difficult to keep in good condition than *Laminaria*, and on this account is not so well suited to investigations of this kind. It demands a low temperature and thorough aeration, and even with these does not live so long as *Laminaria*.

[1] Regarding the composition of the cell wall in algæ see Czapek, F., Biochemie der Pflanzen, Jena, 2nd edition, 1913, i, 640 ff.

[2] Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxvi, 557.

In *Ulva* the structure of the frond is much simpler than in the other plants studied. It consists of two layers of similar cells, forming a membrane about 0.078 mm. in thickness. The cell walls are thin and consist of cellulose.[3] As it is not sufficiently stiff to be treated like *Laminaria*, pieces were cut out and supported between disks of hard rubber and celluloid as described in a previous paper.[4]

The leaves of *Zostera* have a very simple structure. The fibrovascular bundles are poorly developed. There are intercellular spaces, but it is possible to find leaves in which there is very little gas in these spaces. In such material the amount and position of the gas seems to remain unchanged during the experiment. The cell walls are of cellulose.

As the leaves are not wide enough to furnish disks such as were used in other experiments, a different procedure was adopted. A number of pieces were held in a horizontal position (parallel and a little separated) by means of two parallel celluloid combs placed at right angles to the length of the leaves. The electrode carriers moved between the combs, forcing the leaves together when measurements were made. The electrode carriers were held in frames as in the case of *Laminaria*, but the two upper glass rods were removed and a special form of hard rubber plate was placed at each end.

In some of the experiments pieces of the leaf were placed between disks of hard rubber and celluloid, just as in the case of *Ulva*.

In all cases the resistance was first determined in sea water, which was then replaced by another solution of the same conductivity. All readings were made at the same temperature or, if necessary, corrected to the proper temperature.

Experiments on *Laminaria* have shown that it is possible to follow the progress of death in the same manner as the progress of chemical reactions *in vitro*. For the most part the process follows more or less closely a monomolecular course. For *Laminaria* placed in NaCl 0.52 M, the velocity constant of the reaction at 15°C. was found (as the average of eight experiments) to be 0.00723. For *Rhodymenia* the same number of experiments (at the same temperature) gave K = 0.0009. Hence the rate appears to be about eight times as great

[3] The wall contains a methyl pentosan yielding rhamnose. Cf. Czapek,[1] 641.

[4] Osterhout, *J. Biol. Chem.*, 1918, xxxvi, 563, fig. 3.

in the case of *Laminaria*. In *Ulva* the rate is slower than in *Rhodymenia*, but *Zostera* is in this respect intermediate between *Laminaria* and *Rhodymenia*.

Up to a certain point the death process in *Laminaria* is reversible. Thus tissue remaining in NaCl 0.52 M until it has lost 10 per cent of its resistance will recover if replaced in sea water. This is also true of *Ulva*, *Rhodymenia*, and *Zostera*.

According to their effect upon *Laminaria* substances may be divided into two classes: (1) those which (like NaCl) cause only a fall in resistance, and (2) those which (like $CaCl_2$) cause a rise of resistance followed by a fall. There are great differences among these substances in respect to the effects which they produce, but as far as the experiments have gone these differences are similar in all the plants studied. Thus it is found that the amount of rise in resistance and its duration are much less in the case of Mg than of Ca and this applies to all the plants mentioned.

Rhodymenia agrees with *Laminaria* in showing a rise in resistance[5] in $CaCl_2$, $BaCl_2$, $SrCl_2$, $MnCl_2$, $NiCl_2$, and a greater rise in alum, $Ce(NO_3)_3$, and $La(NO_3)_3$.

Ether produced a rise in *Laminaria* and *Ulva* (*Zostera* was not studied). In *Rhodymenia* ether (2.5, 3, 5, and 5.5 per cent by volume), chloroform (0.02, 0.03, and 0.05 per cent by volume), and alcohol (1, 3.5, 7, 8 per cent by volume) added to the sea water produced little or no rise. This is not surprising in view of the fact that these substances always produce less rise in *Laminaria* than does Ca and that even Ca produces very little rise in *Rhodymenia* (Fig. 1). In respect to recovery from the injury caused by these substances *Rhodymenia* agrees with *Laminaria* in that recovery is practically complete in alcohol (if the fall in resistance has not gone too far), but is almost entirely absent in ether and chloroform.[6]

In general it was found that substances of one class antagonize those of the other, not only in the experiments on *Laminaria*,[7] but also in the cases of the other plants mentioned. As was to be expected, the most favorable proportions were not always the same for

[5] Osterhout, *Bot. Gaz.*, 1915, lix, 317, 464.
[6] Osterhout, *Bot. Gaz.*, 1916, lxi, 148.
[7] Osterhout, *Science*, 1915, xli, 255.

the different plants. Thus it was found that in the case of *Rhodymenia* it required more Ca to antagonize Na than it did in the case of *Laminaria*. It was also observed that in the case of *Rhodymenia* (Fig. 2) the antagonism was not so great as in *Laminaria* and this appears to be correlated with the fact that less decrease of permeability is produced by Ca in *Rhodymenia* (Fig. 1). In other words, the effect of such a substance as Ca upon permeability not only indicates

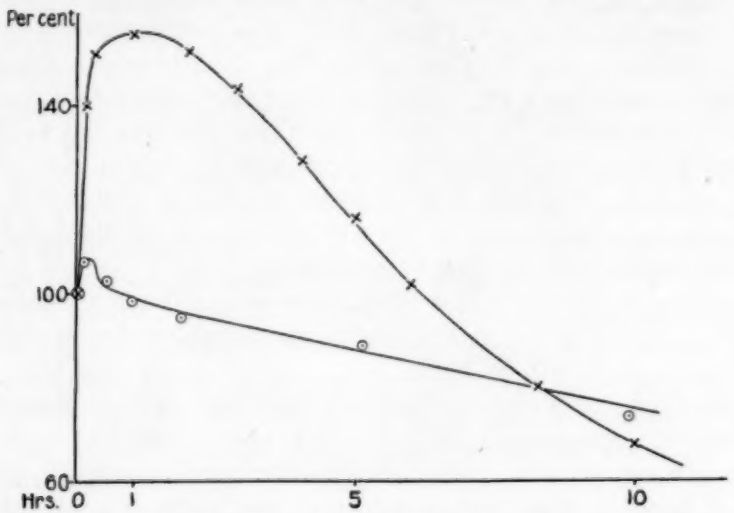

FIG. 1. Curves showing the effect of $CaCl_2$ 0.278 M on the electrical resistance of *Laminaria* (upper curve) and of *Rhodymenia* (lower curve). The ordinates denote net electrical resistance expressed as per cent of the normal resistance in sea water (which is taken as 100 per cent). Temperature 17° ± 2°C. Average of six experiments. Probable error less than 3 per cent of the mean.

what substances it will antagonize, but also, to some degree at least, the amount of antagonism.

It may be added that *Rhodymenia* affords an interesting confirmation of the value of the electrical method in measuring antagonism since the plants begin to change color soon after injury occurs. It was found that the relative rates of death as indicated by color changes in NaCl, $CaCl_2$, and the various mixtures corresponded with the results obtained by determining conductivity.

One of the most interesting experiments on *Laminaria* consists in placing it first in NaCl (causing a fall in resistance) and then in $CaCl_2$, causing a rise. In this way rapid changes of permeability may be produced and the alternation may be continued for a considerable

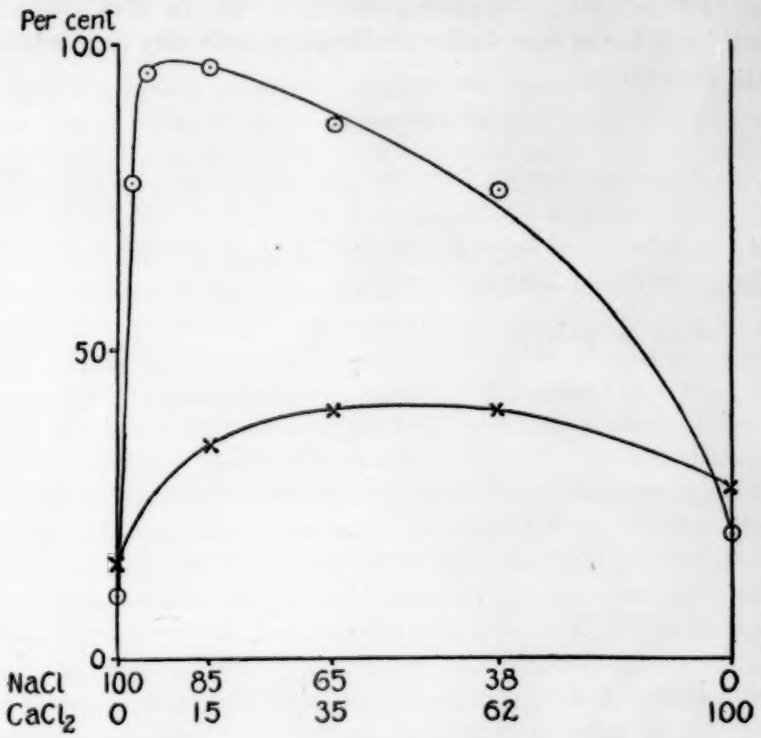

FIG. 2. Curves showing antagonism (after an exposure of 24 hours) between NaCl 0.52 M and $CaCl_2$ 0.278 M in *Laminaria* (upper curve) and *Rhodymenia* (lower curve). The ordinates denote net electrical resistance expressed as per cent of the normal resistance in sea water (which is taken as 100 per cent). The abscissæ denote molecular proportions of the solutions (all the solutions having the conductivity of sea water). Thus NaCl 85, $CaCl_2$ 15 signifies a mixture of 75 cc. NaCl 0.52 M + 25 cc. $CaCl_2$ 0.278 M in which the molecular proportions of Na to Ca are as 85 to 15. Temperature 17.5° ± 5°C. During the 24 hours the resistance of *Laminaria* in sea water remained practically unaltered while that of *Rhodymenia* fell to 84.5 per cent. Average of six experiments. Probable error less than 5.2 per cent of the mean.

period without any bad after effects.[8] This is also the case with the other plants studied.

It appears that in all the plants investigated there is a striking agreement in essentials, though there is considerable diversity in details. It is therefore evident that the conclusions drawn from the study of *Laminaria* are of general validity for all the plants investigated. It is hoped that similar studies on animals may be presented in the near future.

SUMMARY.

Quantitative studies on *Laminaria* (a brown alga), *Ulva* (a green alga), *Rhodymenia* (a red alga), and *Zostera* (a flowering plant) show that the behavior of these plants, in respect to changes in permeability, is essentially alike in all cases.

[8] Osterhout, *Bot. Gaz.*, 1915, lix, 242.

NATURE OF THE RETARDING INFLUENCE OF THE THYMUS UPON AMPHIBIAN METAMORPHOSIS.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

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There is a general agreement concerning the influence of the thyroid upon the metamorphosis of anuran amphibian larvæ. All authors who reported experiments on feeding thyroid to tadpoles, regardless of the locality in which they were carried on and on whatever species of anura they were performed, invariably found that thyroid feeding always resulted in a precocious metamorphosis. The experiments on thymus feeding, however, are characterized by just the opposite feature, the results being very inconstant. Thymus feeding sometimes resulted in retardation or entire prevention of metamorphosis; sometimes it had no effect as compared with the controls. Such different action not only was obtained in the experiments of different authors, performed on different species and at different places (Gudernatsch obtaining positive, Swingle negative results), but even when the same investigator experimented on the same species different individuals were affected differently by the thymus diet (Romeis). Nevertheless, it is certain that in some cases thymus feeding actually prevented metamorphosis and resulted in giant larvæ, while no such effect was obtained in the controls on the normal diet.

It seems necessary therefore to explain why thymus feeding sometimes does and sometimes does not prevent metamorphosis.

The writer performed a large number of experiments on the larvæ of *Ambystoma maculatum*,[1] *Ambystoma opacum*, and *Ambystoma tigrinum*, and it was found that while the thyroid contains a specific substance enforcing metamorphosis, the preventive effect of thymus feeding is due to the absence from the thymus of a substance necessary for the formation or excretion by the thyroid of the substance causing metamorphosis.

[1] For nomenclature of salamanders see Stejneger, L., and Barbour, T., A check list of North American amphibians and reptiles, Cambridge, 1917.

Inhibitory Effect of the Thymus.

Some experiments may be reported briefly showing that thymus feeding sometimes actually prevents metamorphosis, though in the majority of the larvæ it has no such effect.

In a set of larvæ of *Ambystoma maculatum*, kept at approximately 15°C., fed on tubifex and earthworms, and consisting of about 100 specimens, most of the larvæ, which had hatched in the beginning of May, 1916, metamorphosed during the months of August and September. On October 21, eight of these were still larvæ. These were picked out to form a series of normal controls (c. W. 1916). In a set of eighteen larvæ of the same species and of the same age and kept under the same conditions as the above series, but fed on thymus soon after hatching, nine larvæ had metamorphosed up to October 21. The remaining nine were picked out to form a thymus-fed series (c. T. 1916). The worm-fed larvæ of Series c. W. 1916 metamorphosed at an age of 28 weeks and 6 days on an average; the first larva to metamorphose was 25 weeks and 4 days old at the time; the last one 33 weeks and 4 days. Among four larvæ of the thymus-fed Series c. T. 1916, the first animal was 27 weeks old when it metamorphosed, the last one 55 weeks and 2 days. Of these four larvæ the average age at the time of metamorphosis was 35 weeks and 2 days. The other five larvæ of the thymus-fed series died before metamorphosis; three at the age of 31 weeks and 2 days, one at the age of 40 weeks and 3 days, and one at the age of 62 weeks and 3 days. Thus one animal of the thymus-fed series remained larval for about 1 year and 3 months; at this time it did not show any signs of metamorphosis and it seems possible that it would have remained permanently in a larval state. The writer so far has never observed in his normal controls individuals which remained larval for so long a time. Thus there can be no doubt that in this thymus-fed series metamorphosis was retarded as compared with the controls and in one case probably was even prevented.

In a series of eight larvæ of *Ambystoma opacum*, which were kept at about 25°C. and fed moderately on earthworms, the average time of metamorphosis was 26 weeks; in a series of eight larvæ of the same species of the same age and from eggs of the same female as the

larvæ of the foregoing series, the larvæ were fed on thymus and with the largest possible amount of this diet; otherwise they were kept like the worm-fed controls. The average time of metamorphosis in this series was 17 weeks and 5 days; *i.e.*, less than in the worm-fed series. In this number, however, two larvæ are not included, both of which died before metamorphosis; one of them reached the age of 31 weeks and 2 days, the other 34 weeks and 1 day without having metamorphosed. Thus in this series again a considerable delay (if not an inhibition) of metamorphosis was produced by the thymus diet in two animals.

A third species, *Ambystoma tigrinum*, was experimented on. This species, as mentioned in a recent publication[2] shows the least effects when fed on thymus. Among six animals kept at approximately 25°C. and fed on thymus, the larvæ metamorphosed simultaneously with the controls. Among six other animals kept at approximately 15°C. and fed on thymus, only five larvæ metamorphosed simultaneously with the controls (between 22 and 26 weeks after hatching), while one individual though now over 74 weeks old is still in larval condition. Thus also in this species thymus feeding apparently resulted in a considerable retardation of metamorphosis though only in one individual.

From these experiments on urodelan larvæ it is again evident that the effect of the thymus is extremely variable. But it is also a fact that in some of the larvæ metamorphosis was retarded by the thymus diet and in two probably completely prevented. Further experiments, therefore, were carried out to determine this point.

Inhibitive Effect of Thymus upon Amphibian Metqmorphosis Is a Deficiency Phenomenon.

It is clear that the considerable variability of the action of the thymus cannot be explained on the assumption that the inhibitive effect of that gland is due to the presence of a specific inhibiting substance in the thymus. But it can be explained if this effect is due to the absence from certain parts of the thymus of a substance necessary to produce metamorphosis and which is contained, in minute quan-

[2] Uhlenhuth, E., *J. Gen. Physiol.*, 1918, i, 23.

tities, in other parts of the thymus, in normal food, and perhaps in the water of certain localities where unsuccessful experiments on thymus feeding have been carried out.

If the preventive influence, which the thymus exhibits in some of the larvæ, is due to the presence of some specific metamorphosis-inhibiting substance, metamorphosis evidently should be prevented by the thymus even if normal food is added to the thymus diet. This is the case, for instance, with the metamorphosis-producing substance of the thyroid gland. Lenhart[3] has shown that if a certain amount of the active substance of the thyroid, able to produce accelerated differentiation and not large enough to result in death from emaciation before differentiation can take place, is introduced into the organism, differentiation will be accelerated and at a definite rate, whether the tadpoles are fed only on thyroid or whether some other food (liver) is added to the glandular diet. In fact, it seems, from all experiments so far performed with thyroid, that it is of no importance what food the larvæ receive; the addition of only a minute quantity of thyroid substance causes metamorphosis at an accelerated rate. We found the same to be true for the larvæ of salamanders. Young (5 weeks old) larvæ of *Ambystoma opacum* which were fed on earthworms, were placed in a 0.02 per cent solution of iodothyrin; in spite of the earthworm diet and of the small quantity of thyroid substance used (Bayer's iodothyrin), all larvæ metamorphosed from 8 to 9 days after the commencement of the thyroid treatment, while the controls needed 7 to 8 weeks more to metamorphose. The thymus itself contains a specific substance which is highly toxic and produces tetany in the larvæ of *Ambystoma maculatum* and *opacum* as described in a former article.[2] The action of this substance as regards its constancy is quite similar to the action of the metamorphosis-producing substance of the thyroid and very unlike the metamorphosis-inhibiting action of the thymus. No matter what food is added to the thymus diet larvæ fed on thymus always had tetany. And like the thyroid substance, the tetany toxin of the thymus is also characterized by the constancy of its action; it produces tetany in each individual.

[3] Lenhart, C. H., *J. Exp. Med.*, 1915, xxii, 739.

It is quite different with the prevention of metamorphosis by the thymus. If normal food is added to the thymus diet, metamorphosis will take place in each individual at the same time as in the controls. This is shown in the following experiments on larvæ of *Ambystoma maculatum* of the same age, from eggs of the same female, and all kept at approximately 25°C. One series of 30 larvæ was fed only on worms, one series of 15 larvæ only on thymus, and one series on thymus and worms alternately. Fig. 1 shows the result. The larvæ

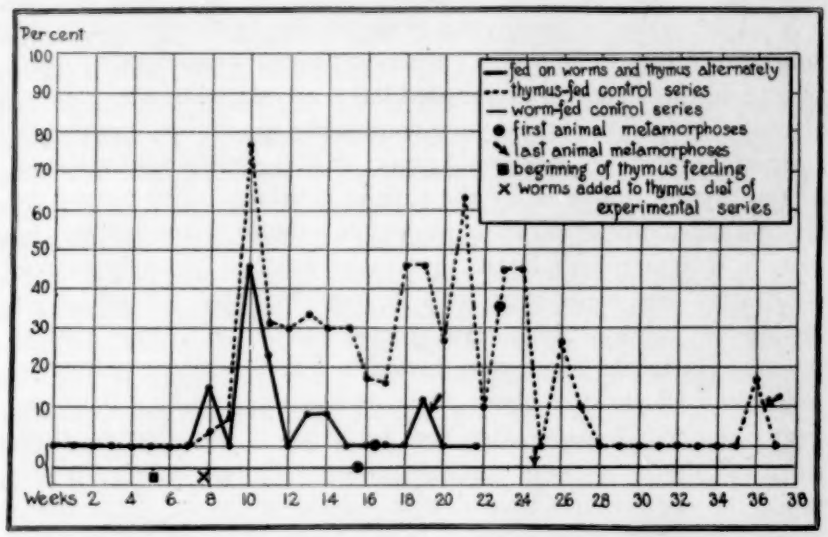

FIG. 1. Effect of worm diet when added to thymus diet upon metamorphosis and tetany in *Ambystoma maculatum* larvæ. Curves indicate percentage of tetanic animals.

of the mixed food series metamorphosed simultaneously with those of the control series, which were fed on worms only; while in the series fed on thymus, metamorphosis started when all animals of the series fed on worms and on worms and thymus alternately had already metamorphosed, and the last larval animal died about 12 weeks later without having metamorphosed at all. In other words, in the series fed on mixed diet the food added to the thymus diet contained the substance which is necessary for metamorphosis and which is lacking in the thymus; and thus this substance was introduced into

the larval organism in such a large amount that metamorphosis occurred in this series simultaneously with the controls. The ability of the normal food to counteract the inhibitive effect of the thymus upon metamorphosis was the more conspicuous as the same food was quite ineffective in preventing the tetanic convulsions produced by the tetany toxin present in the thymus; this may be seen from the curves which indicate the percentage of tetanic animals found at the time of observation among the thymus-fed and mixed food series.[4]

That the action of the thymus is merely due to the absence of the substance necessary to produce metamorphosis, is also demonstrated in experiments in which thymus-fed larvæ are placed in a solution of thyroid substance. If the thymus contained a specific metamorphosis-preventing substance, one would expect an antagonistic neutralization of the thyroid substance by the thymus substance. But instead the thyroid substance even when present in minute quantities induces prompt metamorphosis in the thymus-fed animals. For the sake of illustration one experiment may be reported here. A set of six thymus-fed larvæ of *Ambystoma opacum* was fed on thymus. At an age of 6 weeks the larvæ were placed in a 0.02 per cent solution of iodothyrin which after about 20 hours was replaced by a 0.006 per cent solution. 8 days after the beginning of the thyroid treatment all the larvæ were metamorphosed although fed on thymus, while the controls not treated with iodothyrin needed from 6 to 7 weeks more to metamorphose. In this series again we observe that the effects of the thymus, which actually are due to the presence of a special[5] substance in the thymus, are not stopped by the thyroid treatment. The larvæ exhibited severe tetanic convulsions caused by the tetany toxin of the thymus; these convulsions occurred in spite of the thyroid treatment with undiminished strength.

[4] For detailed discussion see Uhlenhuth, *J. Gen. Physiol.*, 1918, i, 33.

[5] The term specific in connection with the active principle of the thyroid gland has been avoided here, for it does not seem to be proved definitely that the effects exerted by the iodothyrin cannot be brought about by any other substance or any other factor. Since the term specific refers not only to the origin of the inner secretory substances but also to their effects, it is misleading in connection with the thyroid substance.

DISCUSSION.

From the above experiments it is evident that an exclusive thymus diet sometimes can retard or even prevent metamorphosis. But while the ability of the thyroid to enforce metamorphosis is due to the presence in the thyroid of a special substance, the inhibitory action of the thymus is due to the absence of a substance without which metamorphosis is impossible.

In order to appreciate this fact fully we must remember the experiments performed by Allen[6] and Hoskins,[7] which have demonstrated that tadpoles whose thyroid glands have been extirpated are unable to metamorphose. This means that under normal conditions at the time of metamorphosis the thyroid begins to excrete the metamorphosis-producing substance which under experimental conditions is introduced into the organism by feeding thyroid to the larvæ or keeping them in a solution of thyroid substance. Since under normal conditions no thyroid is fed to the animals and since it is the thyroid of the animal itself which excretes the substance in question, we must assume that the normal food of the larvæ contains a substance which is necessary to develop the thyroid of the larva to a state in which it can excrete the metamorphosis-producing substance. Whether or not the substance necessary to develop the thyroid and furnished in normal conditions by the normal food of the larvæ is identical with the metamorphosis-producing substance excreted later on by the thyroid, cannot be decided at present; but in this respect the attempts made by Allen[6] to enforce metamorphosis of thyroidless larvæ by feeding them on thyroid are important. If it is possible to enforce metamorphosis in thyroidless larvæ by feeding them on thyroid, but impossible by feeding them normal food, the substances contained in the normal food are able to develop the thyroid to the excreting stage, but they are unable to evoke metamorphosis in the absence of the thyroid, and, therefore, they are not identical with the thyroid substance. The results so far obtained by Allen point in the latter direction. It is this substance, necessary to develop the secretory stage in the thyroid, which is missing in the thymus.

[6] Allen, B. M., *Science*, 1916, xliv, 755; *J. Exp. Zool.*, 1917–18, xxiv, 499.

[7] Hoskins, E. R., and Hoskins, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 102.

The question arises now in which way do some of the thymus-fed larvæ procure enough of the substance required for the development of the thyroid, while other thymus-fed larvæ are unable to obtain enough of it though all of these larvæ apparently are fed on an equally exclusive thymus diet? Although at present an exact statement on this matter is impossible we must seek its explanation in the fact that evidently the amount required is so small that it was difficult in our experiments so far performed to control the sources which sometimes supplied this substance against our will. As to the actual source of the substance it is possible that the connective tissue constituting the septa between the lobules of the thymus may contain some of it; in fact, this is probable since the septa are in no way a specific tissue like the rest of the thymus. The possibility of some of the larvæ having obtained, by chance, more of the septa than others must be admitted. In this way the great variability of the results of the same author could be explained. There is also a possibility that the water may contain some of that substance; in favor of this would be the fact that some authors, like Swingle,[8] did not obtain any retarding effects at all in their thymus-fed series. Of course, the difference in the reaction of different individuals of the same series of one experimenter can be explained less readily on that possibility.

Our experiments suggest the possible character of the influence of environmental factors on metamorphosis. The problem of amphibian metamorphosis, as well as the problem of internal secretion, assumes a new shape in the light of that fact. On the one hand, it has become clear from the experiments of Allen and his followers that metamorphosis is directly dependent on the action of a certain inner secretory gland of the amphibian larva; on the other hand, it is evident that the development of the secretory stage of that inner secretory gland depends ultimately on certain purely environmental, non-glandular factors. And it is now time that we should recall such attempts as those made by Duméril[9] and von Chauvin[10] to enforce or prevent metamorphosis by purely external non-glandular

[8] Swingle, W. W., *J. Exp. Zool.*, 1917–18, xxiv, 521.
[9] Duméril, A., *Ann. sc. nat., Zool.*, 1867, vii, 229.
[10] von Chauvin, M., *Z. wiss. Zool.*, 1885, xli, 365.

factors; these attempts in the case of von Chauvin doubtless were successful, though the actual relation between the factors employed and the result obtained cannot well be understood at present.

SUMMARY.

1. Though thymus-fed salamander larvæ often metamorphose normally, thymus feeding sometimes retards and in rare cases inhibits metamorphosis completely.

2. The addition of normal food to a thymus diet abolishes the inhibitory effect of the thymus.

3. Addition of a small quantity of iodothyrin leads rapidly to precocious metamorphosis of thymus-fed larvæ.

4. The inhibitory effect of the thymus is not due to a specific inhibiting substance in the thymus, but to the absence from the thymus of a substance required to develop the thyroid to the secretory state.

PARATHYROIDS AND CALCIUM METABOLISM.

By EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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MacCallum and Voegtlin,[1] as well as other authors, have found that in tetany resulting from the extirpation of the parathyroids the Ca content of the blood and the organs (brain) is greatly reduced and that the introduction into the organism of Ca salts, subcutaneously, intravenously, or *per os*, suppresses the tetanic convulsions of the animals operated on. These findings have been confirmed recently by Howland and Marriott[2] in tetany of children. Spontaneous tetany in human beings has apparently the same cause as parathyreoprival tetany, both being due to the non-functioning of the parathyroids. Furthermore, it is known[1] that after parathyroidectomy, tetanic convulsions may be suppressed by bleeding the animals and substituting the amount of blood drawn by an equal amount of salt solution. From the latter fact MacCallum and Voegtlin conclude that in the absence of the parathyroids some toxic substance accumulates in the blood, which normally is antagonized by the parathyroids. They assume further that the toxicity of this substance is due to its ability to combine, in some unknown way, with calcium which it extracts from the organs, causing its excretion and thereby diminishing the Ca content of the blood and organs. MacCallum thinks that the muscular convulsions in tetany are the result of the diminution of the Ca concentration, the function of the parathyroids being to regulate the Ca concentration by antagonizing the toxic substance and thus preventing it from extracting the Ca salts from the body.

As regards the existence of a toxic substance involved in the causation of tetany, the writer has shown that such a substance actually

[1] MacCallum, W. G., and Voegtlin, C., *J. Exp. Med.*, 1909, xi, 118.

[2] Howland, J., and Marriott, W. McK., *Bull. Johns Hopkins Hosp.*, 1918, xxix, 235.

exists and is contained in the thymus gland.[3] In the present article certain experiments will be reported demonstrating that calcium is able to suppress the tetanic convulsions, at least to some extent; the writer, however, was unable to convince himself that this effect upon tetany is characteristic for the calcium and furthermore the experiments in question, though they do not exclude a possible relation between the toxic substance and the calcium, prove conclusively that, as far as the animals used in these experiments are concerned, the tetany toxin, even in the presence of the calcium and in the absence of convulsions, brings about severe lesions of the muscular system resulting probably from lesions of the central nervous system caused by the tetany toxin and not prevented by the calcium.

EXPERIMENTAL.

In order to test the action of Ca lactate upon tetanic animals, a number of larvæ of the salamander *Ambystoma opacum* were fed on thymus and kept at the same time in a solution of Ca lactate in ordinary tap water; another set of larvæ of the same age and from the same female were kept in a solution of Mg lactate of the same concentration as the Ca lactate solution. A series of larvæ from a different female, fed on thymus, but kept in ordinary tap water, served as controls; since differences between larvæ from different females as regards the severity of tetany when fed on thymus are so small as to be negligible, the error introduced by comparing larvæ of different females is very small. For each of the three thymus series one series was kept as control, in which all conditions were the same as in the thymus-fed series, except that small pieces of earthworms instead of thymus served as food. None of the worm-fed control series developed tetany.

I. Thymus-Fed, Untreated Controls (Fig. 1, Curve I).—Six larvae of *Ambystoma opacum* were fed on thymus exclusively. As usual the tetanic attacks began after the larvæ had reached a certain developmental stage[3] and soon reached a maximum. Each single individual came down with tetanic convulsions. When metamorphosis was

[3] Uhlenhuth, E., *J. Gen. Physiol.*, 1918, i, 23, 33.

approached, tetanic convulsions ceased, and after metamorphosis all larvæ were free from it.

Besides the tetanic convulsions the other symptoms of tetany were also present in all animals; the legs and feet became permanently twisted and contracted and the entire body assumed the shape char-

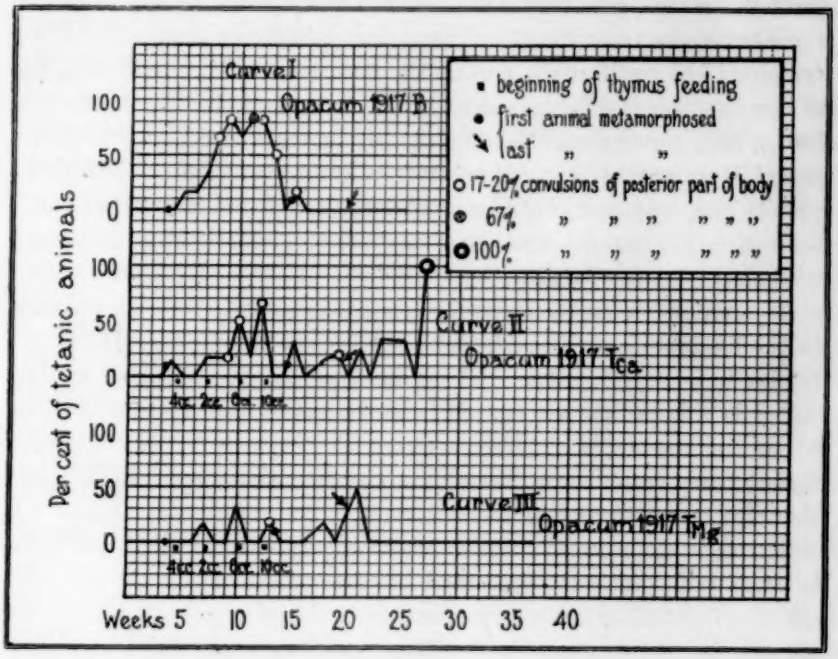

FIG. 1. Tetanic convulsions in a thymus-fed series of *A. opacum* (Curve I); in a thymus-fed series, kept in Ca lactate (Curve II); and in a thymus-fed series, kept in Mg lactate (Curve III). The ordinates indicate the percentage of animals showing tetanic convulsions at the time indicated on the abscissæ. Convulsions of the entire body and convulsions of the posterior portion of the body alone are considered.

acteristic of tetanic animals. The limbs and most of the muscles necessary for the movements of the animals became permanently paralyzed.

II. Thymus-Fed Series, Kept in Ca Lactate (Fig. 1, Curve II).— Seven larvæ of *Ambystoma opacum* were used, one of which died soon after the experiment had been started. Several days after the thy-

mus feeding had been started, one of the larvæ had a tetanic attack. 8 days after the beginning of thymus feeding the larvæ were transferred to a 1/1250 M solution of Ca lactate in tap water; this suppressed tetanic convulsions up to the 7th week, while in the untreated controls tetany had started at the end of the 5th week. Though the concentration of the solution was lowered to only 1/2500 M Ca lactate, the tetany curve did not rise as it did in the controls, but remained low until at the end of the 10th week. The concentration of the solution was increased to 1/625 M, upon which the curve fell, but soon it rose again and, after a further increase of the concentration to 1/500 M, it fell to zero; this latter fall, however, is probably due in part to the approach of metamorphosis, though the rise of the curve soon after the commencement of metamorphosis was due to tetanic convulsions of two larvæ.

As compared with the control thymus series the tetanic convulsions in the Ca lactate series were doubtless somewhat decreased; in particular it is very evident that an increase of the concentration resulted in a fall of the curve. It is worth noting that in the Ca series as in the untreated thymus series each single individual suffered from tetanic convulsions though for a shorter period of its larval life than the larvæ of the thymus-fed, control series.

The most important observation, however, is that though the muscular convulsions were decreased by the action of the Ca salt, the other symptoms of tetany, in particular the permanent paralysis of almost the entire muscular system, developed at the same time and with the same severity as in the untreated thymus-fed control series.

Finally, it should be mentioned that in the Ca series the metamorphosed animals behaved entirely differently from untreated thymus-fed animals. When the larvæ metamorphosed, they were taken out of the solution and placed on moist filter paper. As in all other thymus-fed animals, their muscles were paralyzed and the shape of the body and of the legs was greatly deformed, abnormalities which they had acquired during the tetanic period; like the untreated thymus-fed animals, they did not suffer from tetanic convulsions. Several weeks, however, after metamorphosis tetanic convulsions started again in the Ca animals, in contradiction to what we have observed

in all the untreated thymus-fed animals. This surprising difference has not found so far any explanation and will not be considered in the present article, detailed discussion being reserved until further experiments upon this phenomenon are available.

III. Thymus-Fed Series, Kept in Mg Lactate (Fig. 1, Curve III).— Seven larvæ of *Ambystoma opacum* were used; one of them died soon after the experiment had been started. 8 days after the beginning of the thymus feeding and before tetanic convulsions had made their appearance, the larvæ were transferred to a 1/1250 M solution of Mg lactate in tap water. By this concentration tetanic convulsions were suppressed until the end of the 7th week; at this time one larva had convulsions. But even though the concentration had been lowered to only 1/2500 M, no further convulsions occurred until the 10th week; the concentration was increased to 1/625 M, upon which the curve fell immediately to zero. The concentration was further increased to 1/500 M; only one larva developed tetanic convulsions of the posterior portion of the body, and no further attacks were observed among the larvæ, the rise of the curve at the end of the 18th week being due to tetany of a metamorphosed animal.

In this series the Mg lactate had a distinct and very definite influence upon the frequency and severity of the muscular convulsions; not only is the curve running far lower than in the untreated thymus-fed series, but it is also lower than in the Ca series. Furthermore, three of six larvæ had no muscular convulsions during the larval period. Evidently the effect of the Mg lactate in suppressing the muscular convulsions during tetany is far greater than that of the Ca lactate, when used in the concentrations employed in these experiments.

This influence of the Mg lactate upon the convulsions of the muscles, however, does not mean that Mg is able to suppress tetany, for the other symptoms of tetany, *i.e.* paralysis of the muscles and deformation of the extremities and of the body, develop and to the same degree as in non-treated thymus-fed larvæ.

Concerning the metamorphosed animals the same phenomenon is observed as in the Ca series. A number of weeks after the animals had metamorphosed and were set on moist filter paper without being exposed longer to Mg lactate, they began to suffer again from tetanic

convulsions. And two of the animals which did not have convulsions during their larval period had convulsions after metamorphosis. But the third specimen, which had no convulsions during its larval life, never had tetanic convulsions; it was still alive 43 weeks after metamorphosis.

DISCUSSION AND CONCLUSIONS.

The experiments reported in this article are in full agreement with the facts known about the action of Ca and Mg salts in tetanic animals. In the concentrations used here both Ca lactate and Mg lactate suppressed the muscular convulsions in the tetanic salamander larvæ. The Mg lactate, however, appears to be more effective than the Ca lactate. At any rate the suppression of the tetanic convulsions does not seem to be a specific action of the calcium.

The most important result seems to be the fact that the salts used, though they prevented the muscular convulsions, did not prevent the other symptoms of tetany which in the salamander larvæ are very definite and constant. The permanent spasmodic contractions and the paralysis of the muscles developed in spite of the presence of the Ca and Mg. Furthermore, the muscular contractions and the paralysis developed even in such thymus-fed animals in which the convulsions had been suppressed completely; this was the case in one of the animals of the Mg series.

From the experiments of Biedl[4] and others it is likely that the tetanic convulsions are due to lesions of the central nervous system, since convulsions of a leg can be prevented by isolating it from the central nervous system by cutting the nerves which connect the muscles with the central nervous system. Evidently these lesions of the central nervous system are the chief factor in tetany, while the convulsions of the muscles are only an effect. In the larvæ of salamanders these lesions find a definite expression in the permanent paralysis of almost the entire muscular system.

In the writer's opinion, MacCallum's hypothesis that the tetany toxin has a special affinity for Ca, thereby diminishing the Ca content of the organism, cannot be disproved at present. But the

[4] Biedl, A., Innere Sekretion, Berlin and Vienna, 1913, i, 126.

present experiments seem to prove, first, that the tetany-producing substance causes permanent lesions of the nervous system, which lead to permanent spasmodic contractions and paralysis of the muscle even in the absence of tetanic convulsions, and second, that these cannot be prevented by either Ca or Mg. For the most part they result in an early death of the animals no matter whether or not Ca or Mg has been applied.

In connection with this fact we wish to mention Biedl's claim[4] that no one has yet succeeded in prolonging the life of parathyroidectomized animals by the application of Ca. From MacCallum's paper, on account of the lack of controls, it cannot be seen whether his parathyroidectomized dogs lived longer with Ca treatment than without.

That in spontaneous tetany Ca treatment may effect a cure, as is evident from the report by Howland and Marriott, does not prove that in this case Ca has inhibited tetany as a disease. In spontaneous tetany the period of the action of the tetany-producing substance may be a very short one and the mere prevention of the tetanic convulsions may keep the patient alive until normal function of the glands involved has been restored. The pathological changes which the central nervous system undergoes in this short period may not be severe enough to endanger the life of the patient after the cessation of the action of the tetany toxin.

In the light of the facts presented our experiments lead to the following conclusions:

1. The thymus gland excretes a tetany-producing substance which in the normal animal is antagonized in an unknown way by the parathyroids.

2. In animals devoid of parathyroids (salamander larvæ, parathyroidectomized mammals) this substance may, according to MacCallum, reduce the Ca content of the organism; but by far the most dangerous and important quality of this substance is its highly injurious effect upon the central nervous system, which causes permanent spasmodic contractions of the muscles and paralysis of almost the entire muscular system.

3. It is possible to prevent the muscular contractions by introducing Ca salts into the body, though this can be done more effectively by means of Mg salts.

4. No substance, however, has been found so far to antagonize the tetany toxin and to prevent the development of the lesions of the central nervous system caused by the tetany toxin.

5. This explains why in spite of the application of Ca or Mg and in spite of the suppression by these substances of the tetanic convulsions the other symptoms of tetany develop and frequently lead to the death of the animal.

6. Accordingly the most important function of the parathyroids is to prevent the tetany toxin, by antagonizing it, from coming into contact with the central nervous system.

The writer wishes to express his thanks to Dr. G. M. Meyer, of The Rockefeller Institute, for preparing the solutions used in these experiments.

RATE OF RECOVERY FROM THE ACTION OF FLUORITE RAYS.

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This paper is a report of some experiments upon the rate of recovery of *Paramecium caudatum* from the cytolytic action of fluorite rays. The organisms were exposed to the radiation emitted through the fluorite window of the hydrogen discharge tube described in previous communications.[1,2] The intensity of the radiation was such that an exposure of 8 seconds caused cytolysis in 57 per cent of the exposed organisms. In order to study the rate of recovery from the action of the rays, the entire 8 seconds of radiation was not given in one exposure, but in two portions of 4 seconds each, with a longer or shorter interval of time intervening between the two exposures. The relation between the length of this interval of time and the percentage of organisms cytolized was observed.

The organisms used were from a pedigreed race of *Paramecium caudatum*, cultured in drops of nutrient infusion on concave microscope slides. A single organism was placed in a small drop (measured to uniform size) and exposed to fluorite radiation on a special microscope slide provided with a fluorite window. The rays passed through the fluorite window of the microscope slide from below. After the exposure the small drop containing the organism was flooded with from one to two drops of infusion and the organism was transferred to a new concave slide and placed in a damp chamber for observation. The following day, the cytolized organisms were disintegrated, while the organisms which survived were active and

[1] Bovie, W. T., The action of Schumann rays on living organisms, *Bot. Gaz.*, 1916, lxi, 1.

[2] Hughes, D. M., and Bovie, W. T., The effects of fluorite ultra-violet light on the rate of division of *Paramecium caudatum*, *J. Med. Research*, 1918, xxxix, 233.

had usually undergone fission. From previous experiments[2] on the effects of these rays on *Paramecium caudatum* we have learned that if the organisms are actively swimming 24 hours after the radiation, they will continue to live and will multiply at the same rate as the non-radiated controls. The experimental results are given in Table I.

It will be seen from Table I that as the interval of time between the two exposures increases, the per cent of cytolized organisms decreases. During the time between the two exposures the organism recovers from the effects of the first 4 second exposure so that when the second 4 second exposure is added the total effect is less than that of a single 8 second exposure. The amount of this recovery in-

TABLE I.

Rate of Recovery from the Action of Fluorite Rays.

Interval of time between exposures.	Total No. of organisms exposed.	No. of organisms cytolized.	Cytolysis.		Calculated No. of organisms cytolized.
				Corrected.	
min.			*per cent*	*per cent*	
0	72	41	57.0	50.0	41.0
7.5	47	20	42.7	35.7	19.75
15	54	17	31.5	24.5	17.0
30	49	9	18.3	11.3	9.35
60	50	3	6.0		4.9

After one exposure of 4 seconds the per cent of cytolysis was 7.

creases as the interval of time between the two exposures increases. For example, when the interval of time between the two exposures was 1 hour, the organism had so completely recovered from the effect of the first exposure that the combined effect of the two exposures was no greater than that of a single 4 second exposure.

7 per cent of all the organisms receiving a single 4 second exposure cytolized. If we subtract 7 per cent from each of the percentages given in Column 4 of Table I, we obtain the values given in Column 5. These values are plotted in Fig. 1 as ordinates against the intervals of time as abscissæ. The points fall upon a smooth curve. The shape of the curve suggested that the process of recovery might be adequately represented by the equations which govern homogeneous

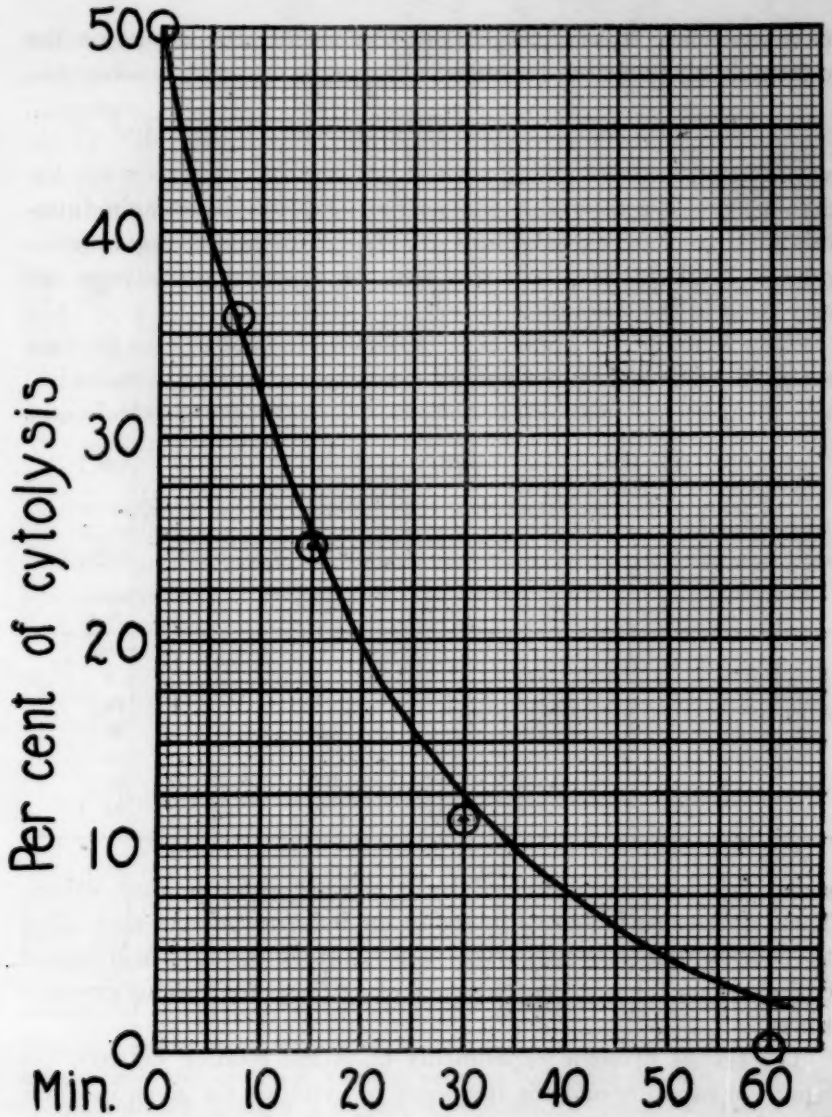

FIG. 1. Graphic representation of the recovery of *Paramecium caudatum* from fluorite radiation as a function of time. The per cents of cytolysis are plotted as ordinates and the intervals of time between the two 4 second exposures are plotted as abscissæ. The curve is a graphic representation of the equation $X = X_0\epsilon^{-Kt}$, in which K equals 0.0473, X_0 equals the per cent of cytolysis when the time interval is zero, and X the per cent of cytolysis after the intervals of time, t. ϵ is the base of the natural system of logarithms.

mass reactions. I have plotted for comparison the curve for the monomolecular reaction formula

$$X = X_0 \epsilon^{-Kt}$$

when K equals 0.0473, X_0 equals the per cent of cytolysis when the time interval is zero, and X the per cent of cytolysis after the intervals of time, t. The calculated number of cytolized organisms is given in Table I. It will be seen that the observed percentages fall very close to the theoretical curve.

When a number of organisms are exposed to fluorite rays they are not all killed by the same length of exposure, but, owing to individual idiosyncrasies and unknown variations in the experimental conditions,

TABLE II.

Relation between the Frequency of Cytolysis and Length of Exposure to Fluorite Rays.

Length of exposure.	No. of organisms exposed.	No. of organisms cytolized.	Cytolysis.
sec.			*per cent*
6	51	1	2
8	100	29	29
10	106	48	46
12	114	79	69
14	120	109	91
16	105	99	94

some organisms are cytolized by a shorter exposure than others. These differences in susceptibility to the influence of rays may affect the shape of the recovery curve. The nature of the effect will depend entirely upon the relative frequency of cytolysis for various exposure times.

The results of some experiments upon the relation between the length of exposure and the frequency of cytolysis are given in Table II and are represented graphically in Fig. 2. It was not possible in these experiments to duplicate all of the conditions such as light intensity, etc., of the recovery experiments; thus a 4 second exposure did not cause any of the organisms to cytolize. The experimental results are, however, significant in connection with the recovery ex-

periments, for as will be seen by an inspection of Fig 2, for exposures between 6 and 14 seconds duration, with but a single exception,

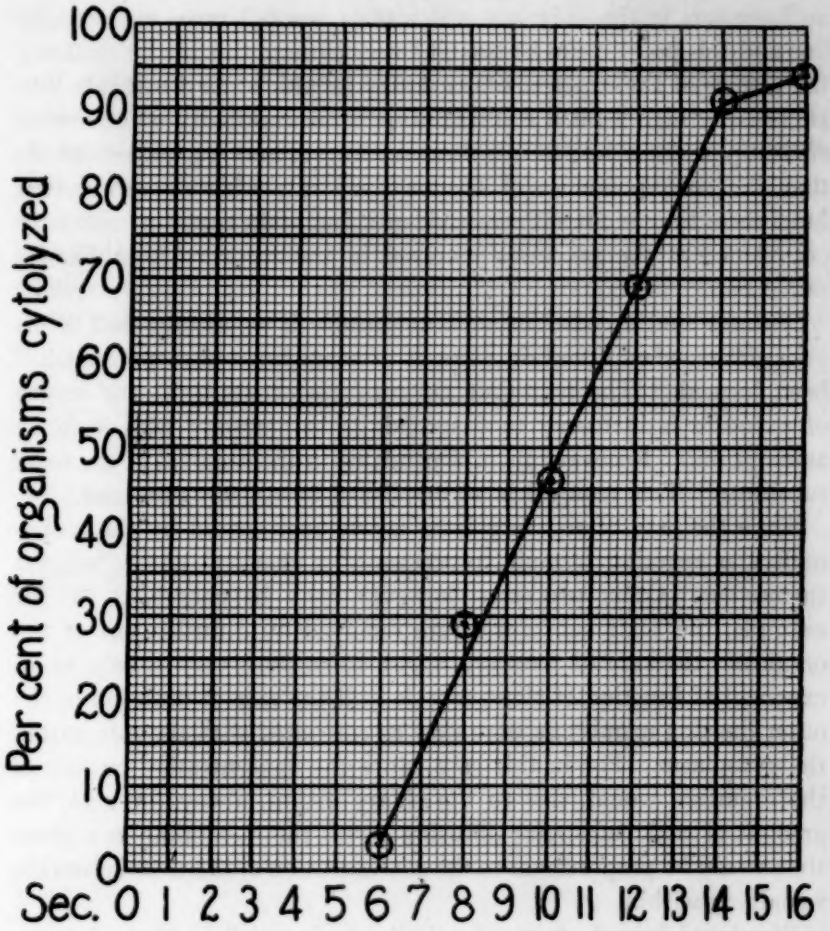

FIG. 2. Graphic representation of the frequency of cytolysis of *Paramecium caudatum* as a function of the length of exposure to fluorite radiation. The per cent of organisms cytolized is plotted as ordinates, the length of exposure as abscissæ.

the curve expressing the relation between the frequency of cytolysis and the length of exposure is practically a straight line. That is, within the limits of experimental error, there are just as many organ-

isms in the class requiring 10 seconds as there are in the classes requiring 12 or 14 seconds exposure to produce cytolysis. The point at 8 seconds is the only one which does not fall upon the straight line and this point could be brought very close to the line by omitting the results of the experiments of 1 day in which the death rate was abnormally high. Such a distribution of susceptibility will have no effect upon the shape of the recovery curve, and we may conclude that the correspondence of the recovery rate with an exponential function of time is not entirely accidental but is the result of an orderly occurrence of the processes involved in the recovery from fluorite radiation.

We may find a cause for such uniformity if we assume that cytolysis occurs when a certain amount of some toxic photoproduct has been formed. (The fact that no cytolysis occurs until the length of exposure is increased to 6 seconds is in harmony with such an assumption.) Recovery then, depends upon the removal of the toxic substance. This removal is accomplished by orderly processes.

Since the rate of recovery is so nearly represented by the monomolecular reaction formula, processes of a chemical nature suggest themselves. Other processes, however, may be conceived. If for example, the toxic substance were removed by diffusion out of the organism, the rate of recovery might correspond very closely to an exponential function of time, especially if the rate of diffusion in the outer limiting membrane were slow as compared with the rate within the cytoplasm. For in this case, since the concentration outside of the organism would, due to the ciliary action, always be zero, the amount of toxic substance diffusing across the membrane in a given time would be proportional to the concentration of the toxic substance remaining within.

Wood and Prime[3] advanced a similar explanation for their observation that it required a much longer exposure to kill carcinoma tissues *in vivo* than *in vitro*. They say[4]: "The constant supply of fresh nutriment to the cells by the blood and the removal of any chemical products formed by the radium in the tissue, must account for this dif-

[3] Wood, F. C., and Prime, F., Jr., The action of radium on transplanted tumors of animals, *Ann. Surgery*, 1915, lxii, 751.

[4] Wood and Prime, *Ann. Surgery*, 1915, lxii, 759.

ference." This suggestion makes the diffusion of toxic substances out of the radiated cell of interest in connection with the toxemias which often follow large doses of either ultra-violet, Roentgen, or γ-radiations.

However, we cannot arrive at very certain conclusions regarding the processes of recovery until these experiments have been repeated with various exposure times, with various radiation intensities, and at various temperatures. Until the data from such experiments are available we may best leave the subject with the simple statement that during the interval of time between the two exposures the organisms recover from the effects of the first 4 second exposure so that when the second 4 second exposure is added the total effect is less than that of a single 8 second exposure. The rate of recovery corresponds very closely to an exponential function of time.

SENSITIZATION TO HEAT DUE TO EXPOSURE TO LIGHT OF SHORT WAVE-LENGTHS.

By W. T. BOVIE and ALICE KLEIN.

(From the Cancer Commission of Harvard University, Boston.)

(Received for publication, October 25, 1918.)

This paper is a report of some experiments on the effect of heat upon organisms which have been exposed to fluorite rays. The experiments have yielded valuable information concerning the mechanism of the action of rays. They show that the exposed organisms (*Paramecium caudatum*) are made extremely sensitive to the influence of heat.

The experiments are of interest because they show the necessity of a careful control of the temperature of the radiated organisms both during and after the radiation. Heretofore investigators have paid but little, if any, attention to temperature control in their radiation experiments. They have, perhaps, considered the matter unimportant, since the rate of most photochemical reactions is independent of temperature. The effect of heat upon the organism which has been made sensitive to heat by radiation must be clearly distinguished from its effect upon the specific photochemical reaction, for in the former case the effect is upon a series of reactions initiated by the exposure and not upon the photochemical change itself.

The heat sensitization resulting from radiation appears to be of general occurrence. It has been found in *Laminaria* exposed to the rays from radium emanation[1] and in egg white exposed to the rays from a quartz mercury-vapor lamp.[2] In the case of the heat-sensitized egg white visible coagulation occurs at a lower temperature than in the non-radiated control. The degree of sensitization increases with the amount of radiation so that with sufficient expo-

[1] Unpublished experiments by the writer.

[2] Bovie, W. T., Temperature coefficient of the coagulation caused by ultraviolet light, *Science*, 1913, xxxvii, 373.

sure coagulation occurs at 0°C. Gelatin, on the other hand, when heat-sensitized by radiation liquefies at a lower temperature than non-radiated gelatin.[3]

The method of experimentation was as follows: By means of a method previously described[4] a single organism from a pedigreed culture of *Paramecium caudatum* was exposed in a small drop of tap water (always of the same size) to the rays emitted through the fluorite window of a hydrogen discharge tube. The drop of water containing the organism was maintained, unless otherwise stated, both before and during the radiation at a temperature of 16–17°C. After the exposure the organism was placed in a larger drop of tap water in the concavity of a microscope slide on the warm plate of an electric stove. The variable junction of a thermocouple (made with No. 30 B. and S. gauge copper and "ideal" wires) was placed in the drop with the organism. The thermocouple circuit contained a galvanometer from which deflections corresponding to 0.1°C. could be read. The temperatures of the dish from which the drop of tap water was taken and the warm plate of the electric stove were so controlled that no change of temperature, capable of being detected by the galvanometer, occurred in the drop containing the organism during the experiment.

No attempt was made to heat all the organisms used in any one experiment to precisely the same temperature. It was sufficient for these experiments to keep the temperature within a certain range. The organisms were exposed to the temperatures indicated below for a period of 60 ± 3 seconds and were then transferred to a culture drop and placed in the damp chamber with the controls for future observation. The experimental results are given below.

Control Experiments.

Experiment 1. Effect of Radiation Alone.—Organisms were radiated at 17–18°C., for 4 seconds and then transferred immediately to culture drops without being placed on a temperature slide.

[3] Unpublished experiments by the writer.

[4] Bovie, W. T., and Hughes, D. M., Rate of recovery from the action of fluorite rays, *J. Gen. Physiol.*, 1918–19, i, 323.

Total No. of organisms radiated	60
No. of deaths	0
Per cent of death	0

Experiment 1 shows that with the light intensities used, an exposure of 4 seconds to fluorite rays is not sufficient to cause death of the organism.

Experiment 2. Effect of Extra Manipulation of Placing on a Temperature Slide and Then Exposing to Heat.—Organisms were radiated for 4 seconds at 17–18°C., and then exposed on a temperature slide to 16–17°C. for 60 seconds.

Total No. of organisms radiated	30
No. of deaths	3
Per cent of death	10

We had observed that some of the radiated organisms appear to be sticky and it was thought that a certain per cent of the deaths in Experiment 5 might be due to accidental physical injury incurred during the transfer to and from the temperature slide. Experiment 2 gives the result of an investigation of this question.

Experiment 3. Effect of Heat Alone.—Unradiated organisms were exposed on a temperature slide to 24–28°C. for 60 seconds.

Total No. of organisms	21
No. of deaths	0
Per cent of death	0

The results obtained in Experiment 3 were to be expected. They are in agreement with those of Woodruff and Baitsell[5] on the temperature coefficient of the rate of reproduction of *Paramecium aurelia*. According to these authors the optimum temperature zone for reproduction is between 24° and 28.5° C.

Experiment 4. Effect of Heating First and Then Radiating.—Organisms were exposed on a temperature slide to 24–26°C., for 60 seconds, and then radiated for 4 seconds at 17–18°C.

Total No. of organisms radiated	52
No. of deaths	4
Per cent of death	8

[5] Woodruff, L. L., and Baitsell, G. A., The temperature coefficient of the rate of reproduction of *Paramæcium aurelia*, *Am. J. Physiol.*, 1911–12, xxix, 147.

Experiment 4 was made in order to determine whether or not we are dealing with an additive effect instead of heat sensitization.

Sensitization Experiment.

Experiment 5. Effect of Radiating First and Then Heating.—Organisms were radiated for 4 seconds at 17–18°C. and then exposed on a temperature slide to 24–26° C. for 60 seconds.

Total No. of organisms radiated	44
No. of deaths	29
Per cent of death	66

Summary.

Experiment No.	Treatment.	Deaths.
		per cent
1	Radiation alone	0
2	Extra manipulation	10
3	Heat alone	0
4	Heating first then radiating	8
5	Radiating first then heating	66

In a previous paper[4] it has been shown that *Paramecia* recover in 1 hour from the effects of a 4 second exposure to fluorite rays, so that an additional 4 second exposure does not increase the effects of the first.

We were interested to see whether the organisms would likewise recover from the heat sensitization caused by an exposure of 4 seconds to fluorite rays. In the following experiments the organisms were not exposed to the higher temperatures immediately after radiation, but in order to permit recovery to take place were kept at the radiation temperature for varying periods of time.

Recovery Experiments.

Experiment 6. Recovery Period ½ Hour.—Organisms were radiated for 4 seconds at 17–18°C., and after ½ hour at 17–18°C. were exposed on a temperature slide to 24–28°C. for 60 seconds.

Total No. of organisms radiated	20
No. of deaths	4
Per cent of death	20

Experiment 7. Recovery Period 1 Hour.—Organisms were radiated for 4 seconds at 17–18°C., and after 1 hour at 17–18°C. were exposed on a temperature slide to 24–28°C. for 60 seconds.

Total No. of organisms radiated	21
No. of deaths	1
Per cent of death	5

Experiment 8. Recovery Period 5 Hours.—Organisms were radiated for 4 seconds at 17–18°C., and after 5 hours at 17–18°C. were exposed on a temperature slide to 24–28°C. for 60 seconds.

Total No. of organisms radiated	24
No. of deaths	0
Per cent of death	0

The organisms had nearly recovered from the heat sensitization caused by the exposure to fluorite rays in 1 hour, and had completely recovered in 5 hours. In this ability to recover the heat-sensitized *Paramecium* differs from the heat-sensitized egg white, since the latter does not recover however long the interval of time between the radiation and the exposure to heat.

The results of these experiments are clear cut, and show that *Paramecia* which have been exposed to fluorite radiation are so sensitized to heat that they are unable to withstand, even for 60 seconds, temperatures which are optimum for non-radiated controls.

Woodruff and Baitsell[5] found normal *Paramecia* from a pedigreed culture able to withstand a temperature of 32° for about 2 days.

In the experiments on egg white, referred to above, it was shown that egg white which is maintained at 0°C., during the exposure to ultra-violet light, will, with the proper amount of exposure, coagulate when it is warmed to room temperature. On the other hand, egg white which is maintained at room temperature while receiving an equal amount of exposure coagulates during the radiation. Now in this case it might not be improper to say that the coagulation is in reality not photocoagulation but is heat coagulation. The egg white has been heat sensitized by the radiation so that it can no

longer withstand normal room temperatures. We might, perhaps, with equal propriety say that the death of *Paramecia* following fluorite radiation is in reality a destruction by heat. The organism, as a result of the exposure, has been sensitized to heat so that it is no longer able to withstand its normal optimum temperatures.

This statement is made in order to emphasize the importance of heat sensitization rather than to suggest an explanation of the nature of the action of rays. Nevertheless, no theory of the action of rays can be complete which does not take the results of such experiments into consideration.

THE PHYSIOLOGICAL BASIS OF MORPHOLOGICAL POLARITY IN REGENERATION. I.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 21, 1918.)

INTRODUCTION.

In two preceding papers[1] on regeneration in *Bryophyllum calycinum* it had been shown that the mass of shoots formed in a leaf or in a piece of stem to the base of which a leaf is attached increases with the mass of the leaf. This proves that regeneration is a phenomenon of the order of nutrition and growth.

The question arises how this conclusion harmonizes with the well known fact of the polar character of regeneration. When we cut out a piece from a higher plant or a lower animal the piece regenerates, as a rule, a shoot or head at the apical end and roots or a tail at the basal end. In a preliminary paper,[2] published a year ago, the writer described experiments suggesting that the polar character of regeneration might be due to the existence in the circulating sap (or lymph and blood) of inhibitory substances which prevent dormant buds or resting cells from growing out even if an adequate quantity of foodstuffs is available. It was shown by experiments on *Bryophyllum calycinum* that the leaves as well as the growing shoots have an inhibitory influence upon the growth of all the dormant buds situated more basally in the stem. If we assume that these inhibitory influences are due to certain constituents in the sap sent out by growing buds and by leaves, we come to the following theory of the polar character of regeneration. When we cut out a piece of stem from *Bryophyllum* and remove all the leaves, inhibitory substances will continue to flow in a basal direction in the stem. Since the apical

[1] Loeb, J., *Bot. Gaz.*, 1918, lxv, 150; *J. Gen. Physiol.*, 1918, i, 81.

[2] Loeb, *Science*, 1917, xlvi, 547.

region of the piece will be the first to become sufficiently free from these substances, the buds situated at this end will be the first to grow out into shoots. As soon as this happens the new shoots will in their turn send out inhibitory substances in a basal direction in the stem, thereby preventing the growth of the more basally situated buds. In this paper we shall present some of the qualitative evidence for the inhibitory effect of a leaf upon shoot formation, leaving the discussion of experiments of a more quantitative character for a future paper.

FIG. 1. Diagram showing arrangement of shoot buds in the stem of *Bryophyllum calycinum*. The line connecting the two buds in one node is always at right angles to the line connecting two buds in the next node.

We shall deal chiefly with the regeneration of shoots in the stem of *Bryophyllum calycinum*, which can proceed only from definitely located buds. In the axil of each leaf of a stem there exists one bud capable of giving rise to a shoot, which, however, never does so unless the plant is mutilated. Each node of a plant has two leaves in opposite position, and the axis connecting the two axillary buds in one node is always at right angles with the axis connecting the two buds of the next node (Fig. 1). Thus the line connecting the two buds at Node 2 (Fig. 1) is at right angles with the line connecting the two buds in Nodes 1 and 3, etc. The lower leaves in a stem fall off in time, leaving their axillary buds exposed. No other element of the stem except the two buds in each node is capable of growing into shoots.

The elements capable of giving rise to roots are not confined to the nodes but exist all over the stem in a definite layer of the cortex. It can be shown that the sap from the leaf flowing towards the base of a stem favors the growth of roots and inhibits the growth of shoots.

Experiments on Potted Plants.

When we cut off the top of a potted plant of ***Bryophyllum calycinum***, leaving a stem containing only two leaves at the apical node, none

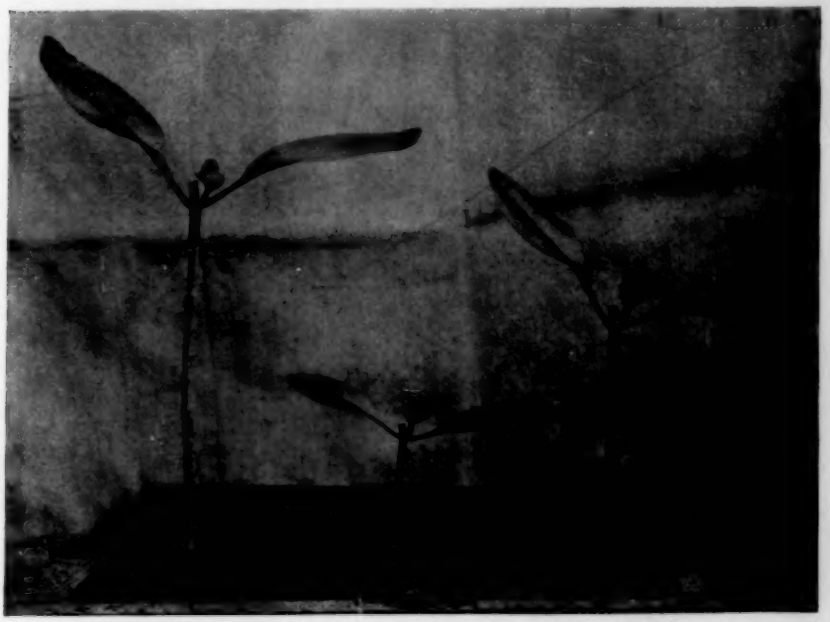

FIG. 2. Top of plant removed; two leaves at apex which prevent growth of all the buds below.

of the buds on the stem below the two leaves will grow out (Fig. 2), so long as the leaves are alive and able to send their sap to the base of the plant. The buds situated in the axil of the two leaves may after some time grow out. The two leaves inhibit therefore the growth of all the buds situated more basally (Fig. 2). Each leaf inhibits the growth of the buds situated in the same half of the stem, and in order to prove this we remove in a second set of experiments

the top of a number of potted plants leaving only one leaf at the apex (Fig. 3). We must also remove the free bud opposite this leaf, since otherwise this bud will grow out and produce the same inhibitory effect as the removed leaf would have done. In this case the experiment would only be a repetition of the preceding experiment in which two leaves were left at the apex. When, however, we remove one apical leaf with its axillary bud very often the one leaf left at the top suffices to suppress regeneration in the basal part of the stem

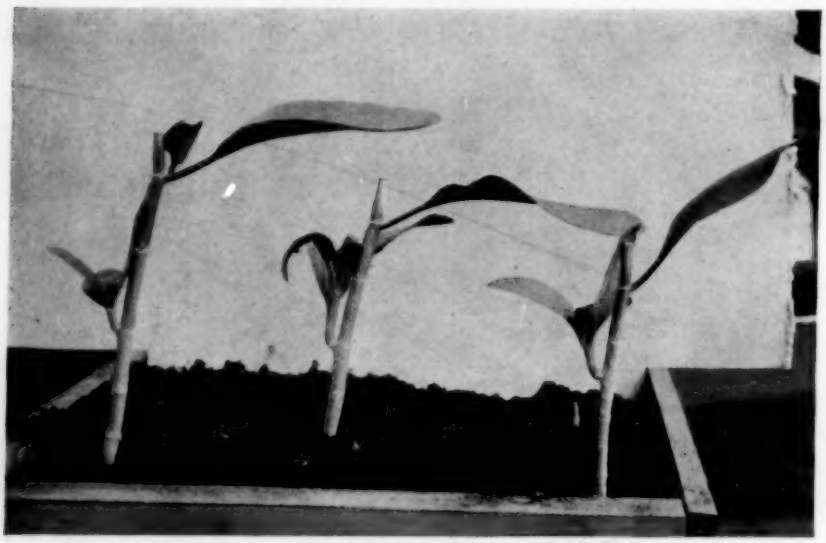

FIG. 3. One leaf left at apex. Growth of shoots in the first node below the leaf suppressed, while the shoot in the second node below the leaf, but on the opposite side of the stem can grow out.

as completely as if two leaves had been left. If regeneration occurs, it takes place in the highest node on the opposite half of the stem which is the second node below the leaf (Fig. 3). In the first node below the leaf no bud can grow out. I have never noticed an exception to this rule in a normal plant. A possible explanation of this phenomenon is furnished by Fig. 4, where that half of the stem through which the sap from the apical leaf flows to the base of the stem is shaded. Since the buds in the first node below

the apical leaf are in the path of the sap flow from the leaf, the formation of shoots is suppressed in these buds, while the bud in the second and fourth nodes below but on the opposite side from the leaf lies outside of the path of the conducting vessels from the leaf. Hence if any bud in such a stem grows out it is usually the one in the second node below but on the opposite side from the apical leaf. As soon as this bud grows out it will inhibit the growth of the lower buds in the same half of the stem.

FIG. 4. Diagram explaining this inhibitory influence of the leaf on the theory that the descending sap from the leaf carries inhibitory substances. The region of the stem reached by the sap from the leaf is shaded and in the shaded part regeneration of shoots is inhibited.

The petiole of a leaf is attached with its base to one-half of the circumference of the stem. When we cut off half of the base of the petiole of a leaf, the sap sent out by that leaf can flow only through one quadrant of the next internode. This should limit the inhibitory influence of such a leaf to this quadrant of the node below, and this turns out to be the case. The top of a number of potted plants was cut off and only one leaf was left at the apex (Fig. 5). Half of the

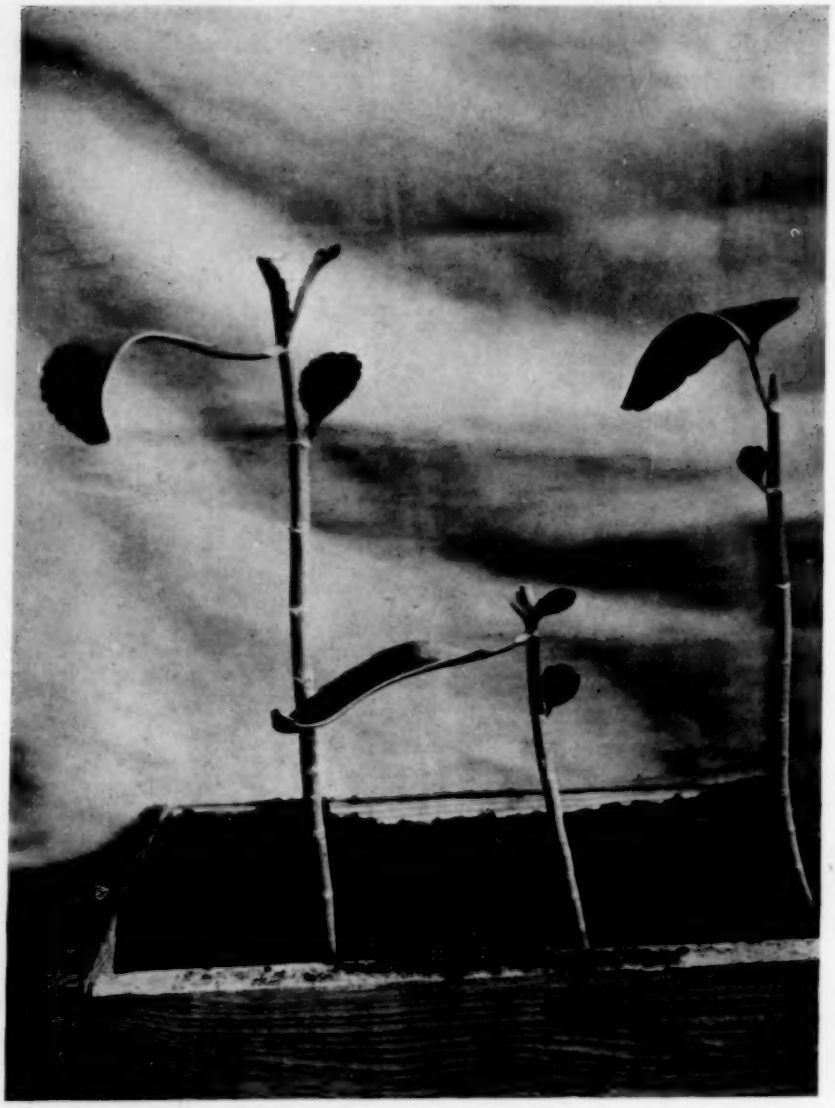

FIG. 5. One-half leaf and one-half petiole left at apex. One of the two shoots in node below leaf now grows out; namely, on the side where half the petiole is removed.

petiole of this leaf was removed at the base and also the corresponding half of the leaf itself was cut off, though this latter procedure is not essential for the result. The axillary bud of the other leaf was also cut out as in the preceding experiment. Fig. 5 gives the result of such an experiment. The reader will notice that in this case one of the two buds in the first node below the leaf will grow out; namely, that one which lies beneath the removed half of the leaf. This bud

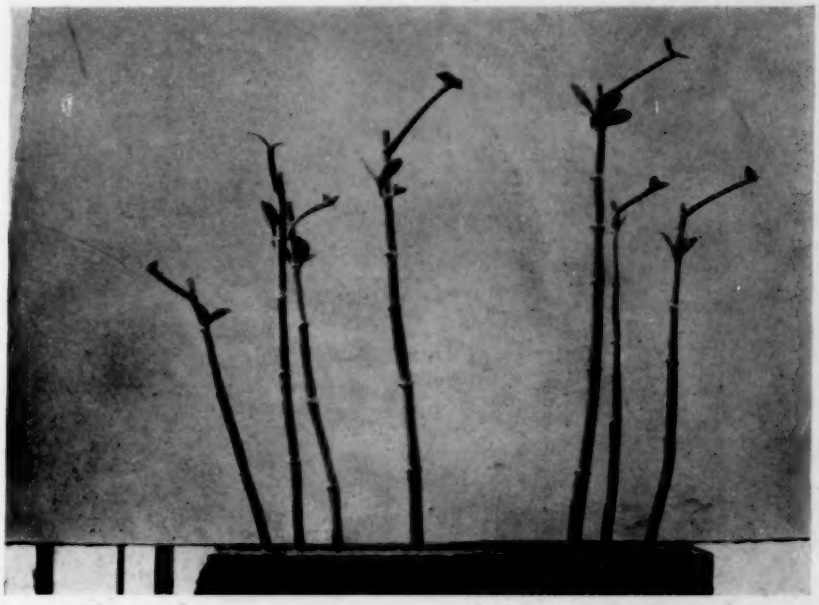

FIG. 6. Showing that the inhibitory influence of an apical leaf upon shoot formation in the node below the leaf disappears when the size of the leaf is sufficiently diminished. Duration of experiment, Oct. 25 to Nov. 14.

grows out since it no longer receives any of the sap from the leaf above. I have never seen the other bud in this node grow out. This experiment also succeeds in practically every case.

We have seen that if we remove one leaf and its axillary bud at the apex of a topped stem, leaving only one leaf at the apex, the buds in the node below are practically always prevented from growing out. When we diminish the mass of the leaf sufficiently (as is done in Fig. 6), this inhibitory influence ceases and every plant forms a shoot in

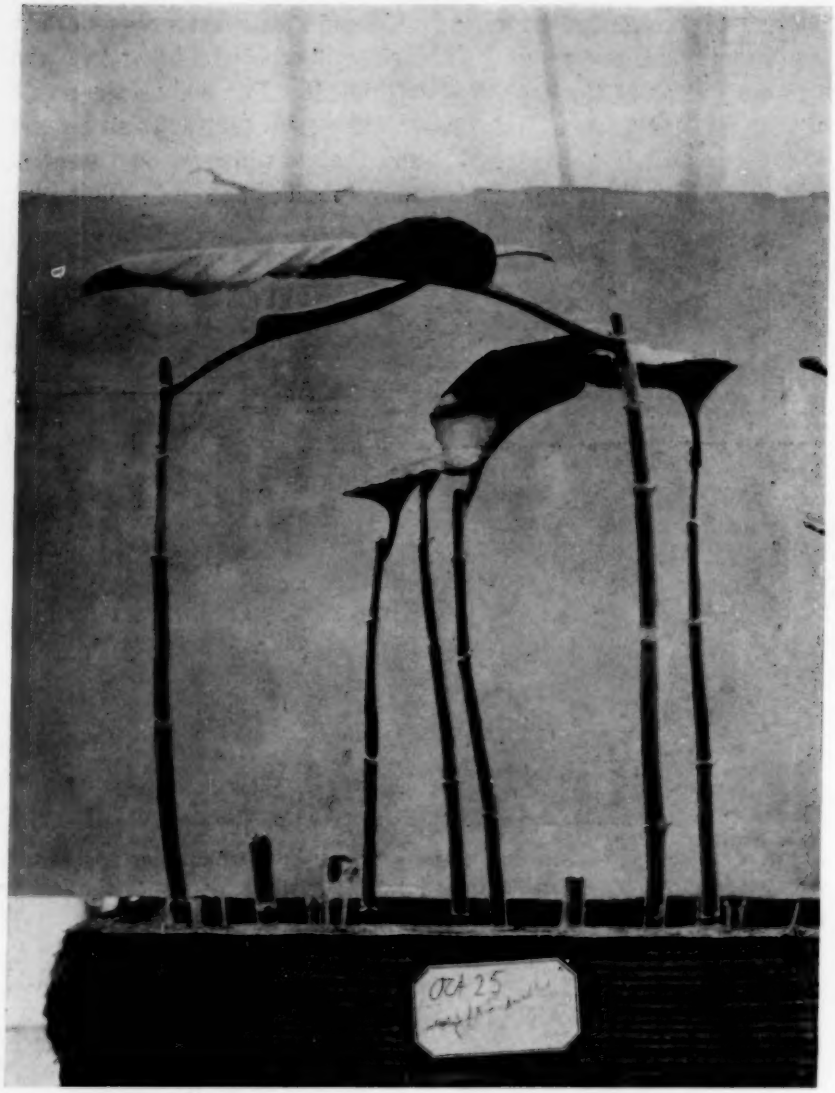

FIG. 7. Control experiment to Fig. 6. Duration of experiment also from Oct. 25 to Nov. 14.

each of the two buds in the first node. Fig. 7 shows the control experiment; namely, six stems each with one whole leaf at the apex. Not a single stem has formed a shoot in the first or any other node below the leaf. Both sets of experiments were carried out simultaneously and both sets of plants were side by side in the same flower bed.

When we reduce the mass of a leaf 10 days after the experiment is started and when the new shoots begin to form, the inhibitory effect nevertheless becomes noticeable.

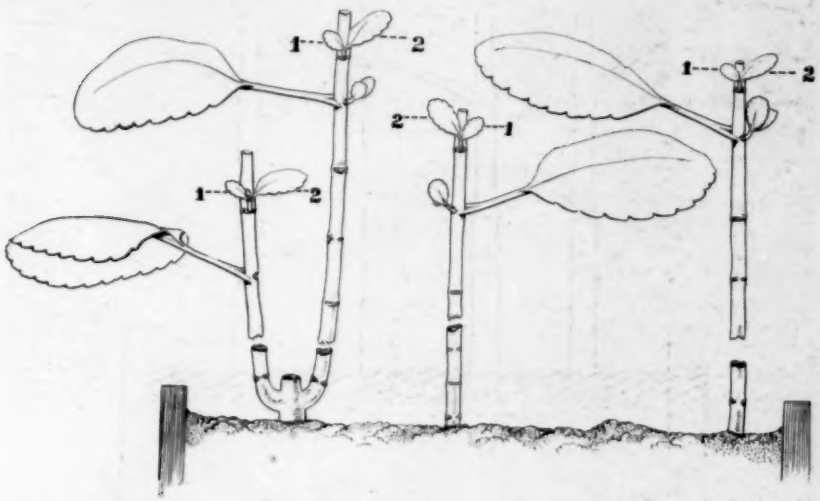

FIG. 8. Proof that traces of inhibition of a leaf upon shoot formation are also noticeable in the more apical shoots. Old leaf left at first node below the apical node. The two Leaves 1 and 2 of the new shoot in the apical node which are normally of equal size show a constant difference, Leaf 1 (on the side where the old leaf is) being smaller than Leaf 2 (on the opposite side).

Demonstration of the Inhibitory Influence of a Leaf on Shoot Formation in a More Apical Node.

We can state as a general rule that a leaf accelerates the growth of shoots at the apex and prevents or retards it in the basal parts of the stem. The leaf has, however, also a slight inhibitory effect on the more apical buds. In order to prove this it is necessary to make experiments like those represented in Fig. 8. In a number of topped

plants one leaf is left at the node below the most apical one. In this case both buds in the most apical node grow out into a shoot giving rise as usual to two small Leaves 1 and 2. While these leaves are normally of equal size, a typical and constant difference exists between the size of the two leaves when one old leaf is left in the node below. Leaflet 1, which has the same orientation as this old and large leaf in

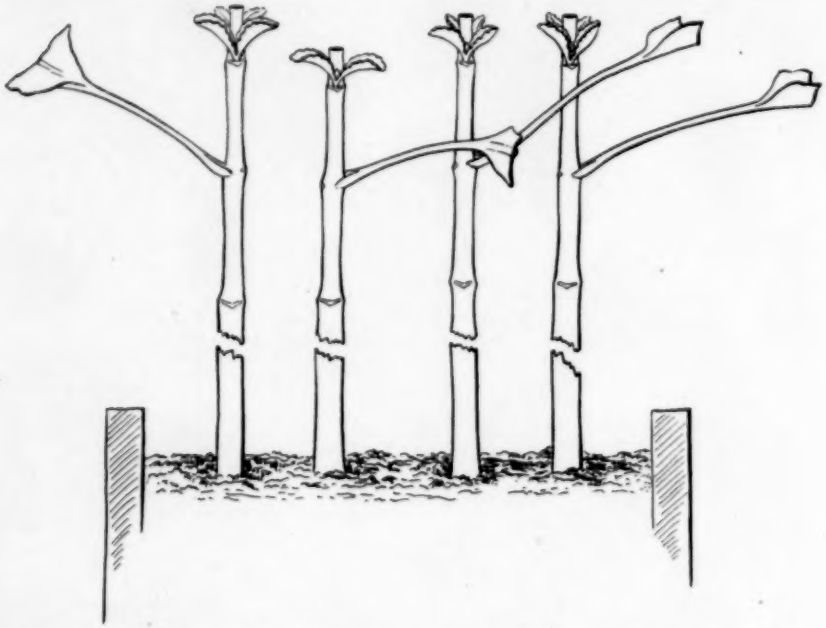

FIG. 9. The inhibitory influence of the leaf upon an apical bud disappears also when the size of the leaf is reduced. Duration of experiment, Oct. 24 to Nov. 7.

the node below, is practically always smaller than the other, Leaflet 2 (Fig. 8). This difference is intelligible on the assumption that a small quantity of the inhibitory substances from a leaf flows towards the apex of the stem; these substances will reach the young leaf facing the same side of the stem where the old leaf is, while they do not reach the other leaf. When we reduce the size of the old leaf, this inhibitory influence disappears (Fig. 9).

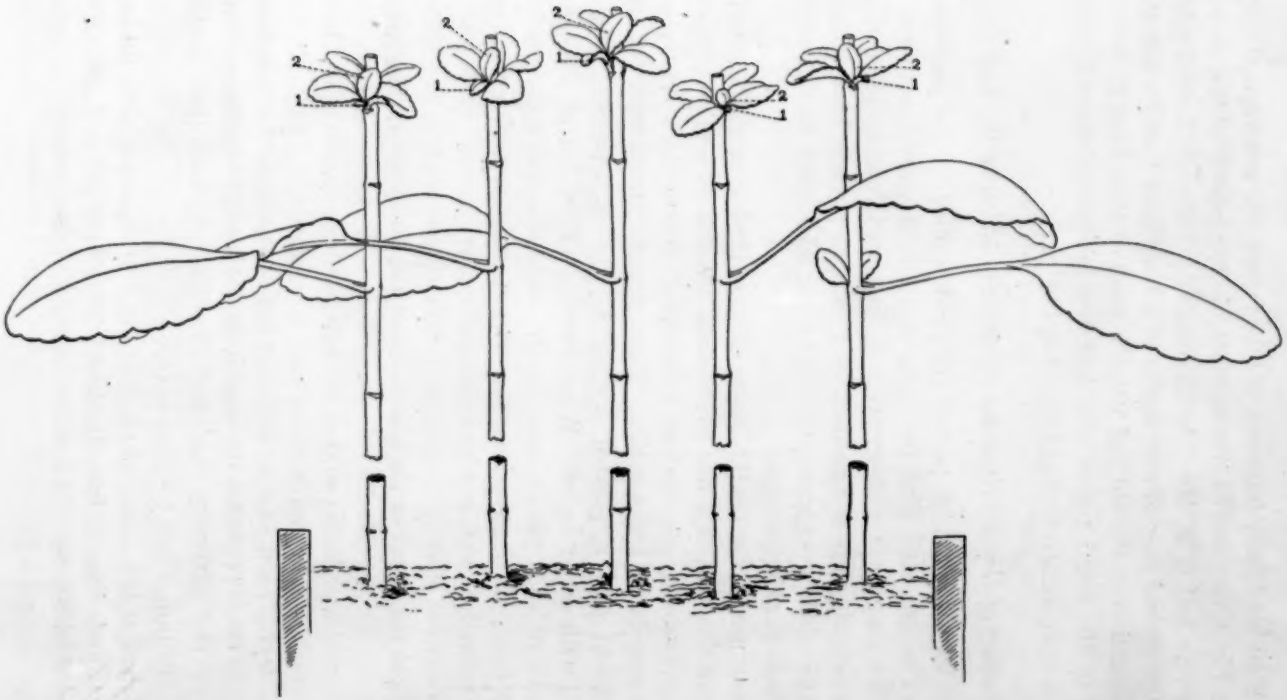

FIG. 10. In the second node above the old leaf the inhibitory effect is shown in the second pair of leaves of the new shoot which are no longer of equal size, Leaf 1 which is above the old leaf being a little smaller than the symmetrical Leaf 2. Duration of experiment, Oct. 17 to Nov. 13.

This slight inhibitory influence of a leaf upon the more apical buds shown in Fig. 8 can also be demonstrated in the growth of the second node above a leaf (Fig. 10). In this case the influence is noticeable only in the second pair of new leaves of a bud; Leaf 1 which has the same orientation as the old leaf remains smaller than Leaf 2.

While in the basal region of a leaf the inhibitory effect is complete, it is comparatively slight in the apical part.

Influence of Gravity upon the Inhibitory Action of the Leaf.

All the experiments on potted plants described in the preceding pages can be repeated with the same result in stems cut out from a plant. We may omit a description of such experiments since they would constitute only a repetition of what has already been stated. But certain of these experiments yield some additional results which are of theoretical importance.

The assumption that the inhibitory effect of the leaf upon the growth of dormant shoot buds is due to chemical substances sent out by the leaf is supported by the striking influence of gravity on regeneration in stems suspended horizontally. Long straight stems were cut out from a plant and suspended horizontally in an aquarium nearly saturated with water vapor. When two leaves are left at the most apical node of such pieces, none of the buds situated more basally will grow out. If, however, one leaf with its axillary bud is removed and the other leaf left, regeneration will occur, but the buds which will grow out will show a characteristic difference according to whether the leaf is on the upper or the lower side of the horizontally suspended stem.

We suspend such stems so that the axis of the two most apical buds (one of which is removed with its leaf) is vertical (Fig. 11). In five stems the leaf is on the upper side and in five stems on the lower side of the stem (Fig. 11). All the stems were originally horizontal but underwent the geotropic bending described in previous papers, whereby the upper side became concave.

When the leaf is below (right half of Fig. 11), shoots may be formed in the first node basally from the leaf. This occurred in three out of five stems drawn here. The other two formed shoots from the upper bud of the second node.

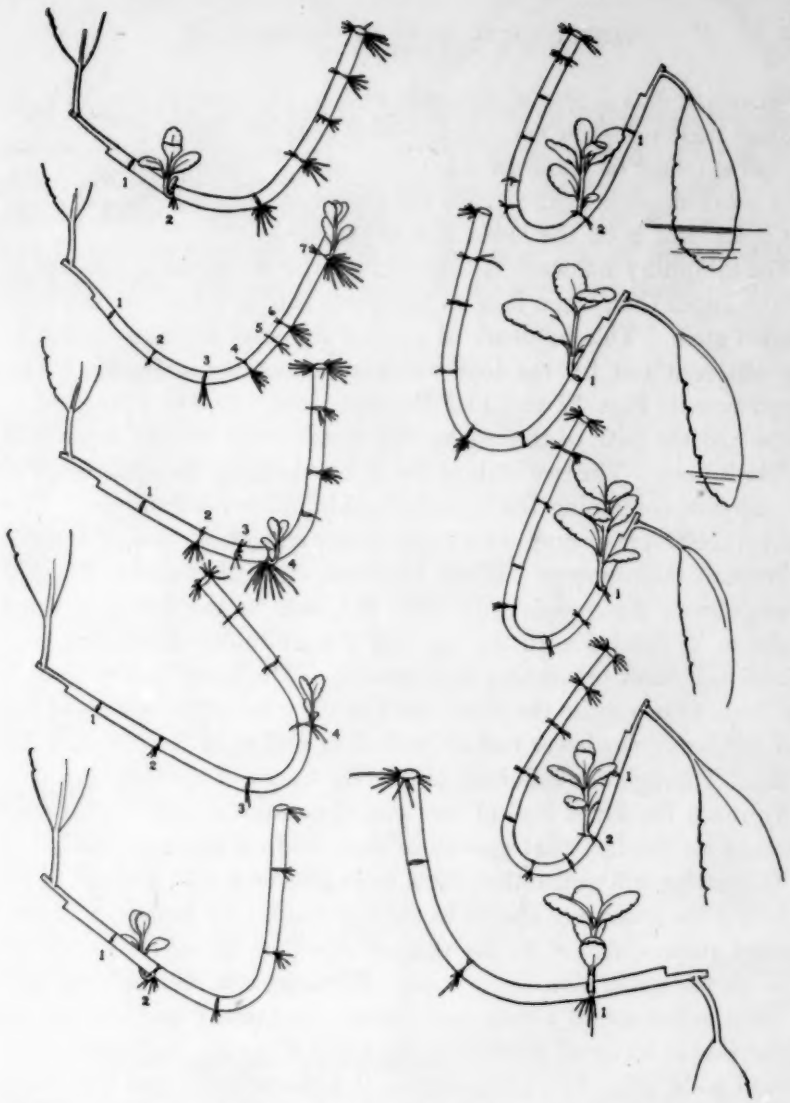

FIG. 11. Influence of gravity on shoot formation in horizontally suspended stems. The stems were originally straight but underwent geotropic bending during the experiment. In the stems to the right one leaf is left at the lower side of apex. In this case the shoots in the first node will grow out in half of the stems; in the second node only the upper bud will grow out. In the stems to the left the leaf is on the upper side. In this case the shoots in the first node are prevented from growing, and only the lower bud in the second and fourth node can grow out. Duration of experiment, Sept. 29 to Nov. 12.

When the leaf is above (left side of Fig. 11) none of the buds in the first node will grow out. If any buds grow out, they are either the second or fourth on the *lower* side of the stem. These shoots grow out with much delay compared with the growth of shoots in stems where the apical leaf is on the lower side of the stem.

The inhibitory influence is therefore greater when the apical leaf is on the upper than when it is on the lower side of a horizontally suspended stem. This influence of gravity supports the idea that it is the sap sent out by the leaf which produces the inhibition. The diagrammatic Figs. 12 and 13 make this clear. In Fig. 12 the leaf is below and the path of the conducting vessels from the leaf is marked by black lines. The two buds of the first node lie on the upper edge of the sap flow containing the hypothetical inhibitory substances The buds of the first node may or may not receive enough of these substances to prevent their growth. When, however, the leaf is above (Fig. 13) seepage from the vessels will cause the buds in the first and third nodes to be flooded with the sap and the inhibitory substances contained in it, thus preventing their growth. The lower bud in Node 2 (or Node 4) is outside the direct path of the conducting vessels of the leaf and hence the lower bud of Node 2 as well as of Node 4 may develop. Through the influence of gravity traces of the sap may possibly reach the lower bud of the second or fourth node. This may account for the fact that growth of these buds is usually retarded.

The reader will notice that these facts give us a neat method of restricting the growth of shoots to the lower side of a horizontally suspended stem, contrary to the general rule that in such cases shoots arise on the upper side of the stem. When we remove the lower half of such a horizontally suspended stem (containing one leaf at the upper side of its apex) leaving on the lower side only the region of the second node (Fig. 14), regeneration of a shoot will occur only from the bud on the under side of this second node. The growth of the buds in the intact upper half of the stem is completely suppressed and the growth of the bud on the under side of Node 2 is slow for reasons stated. No growth will occur on the upper side, except after the leaf is wilted or conduction of its sap through the stem is interrupted.

The correctness of this idea is supported by the further fact that this inhibitory effect of a leaf on the growth of shoots, especially in the basal parts of the stem, is diminished when the mass of the leaf is reduced. Fig. 15 shows such an experiment. Of the five stems on the left each had one whole leaf on the upper side of the originally horizontal stem. In one stem only did a shoot form and this shoot

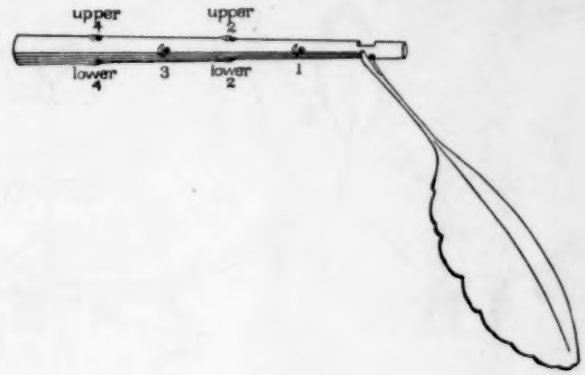

FIG. 12.

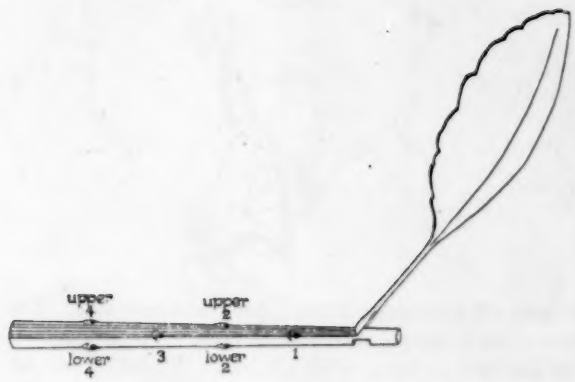

FIG. 13.

FIGS. 12 and 13. Explanation of the influence of gravity on regeneration in horizontally suspended stems on the assumption that inhibition is due to substances carried by the sap sent out by the leaf. When the leaf is below (Fig. 12), the buds in Nodes 1 and 3 are at the upper edge of the sap flow and these buds may or may not escape the inhibitory effect. In Fig. 13 the leaf is above and the sap flowing in the upper half is bound to reach the buds in Nodes 1 and 3 and hence their growth is necessarily suppressed. The lower Buds 2 and 4 are outside the sap flow and may develop.

developed on the lower side in the second node. In the five stems on the right the mass of the leaf was reduced considerably. Four of the five stems formed shoots, two even in the second node on the upper side of the stem. In a repetition of this experiment half of the stems with a reduced apical leaf on the upper side formed shoots in the first node basally from the leaf.

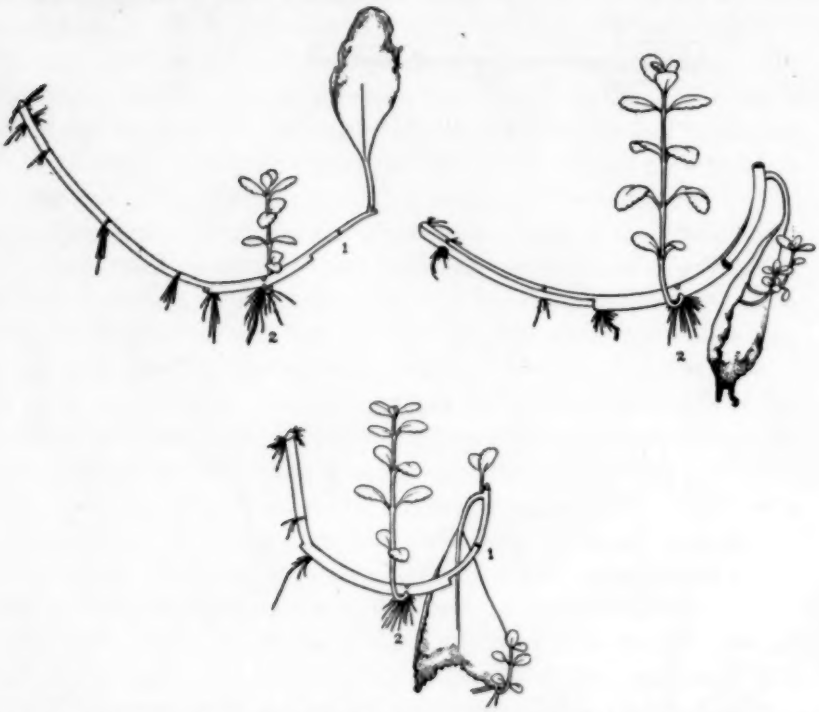

FIG. 14. Horizontally suspended stems. Leaf on upper side. The lower half of stem is removed with exception of second node. The lower bud of the second node grows out into a shoot, while the shoot formation on the upper side of the stem is inhibited by the leaf. Duration of experiment, Feb. 13 to June 24.

The reader will notice that the geotropic bending of the stems was considerably less in the five stems on the right with the reduced leaf than in the five stems on the left with a whole leaf.[3]

[3] Loeb, *Science*, 1917, xlvi, 115; *Ann. Inst. Pasteur*, 1918, xxxii, 1; Forced movements, tropisms, and animal conduct, Monographs on Experimental Biology, Philadelphia, 1918.

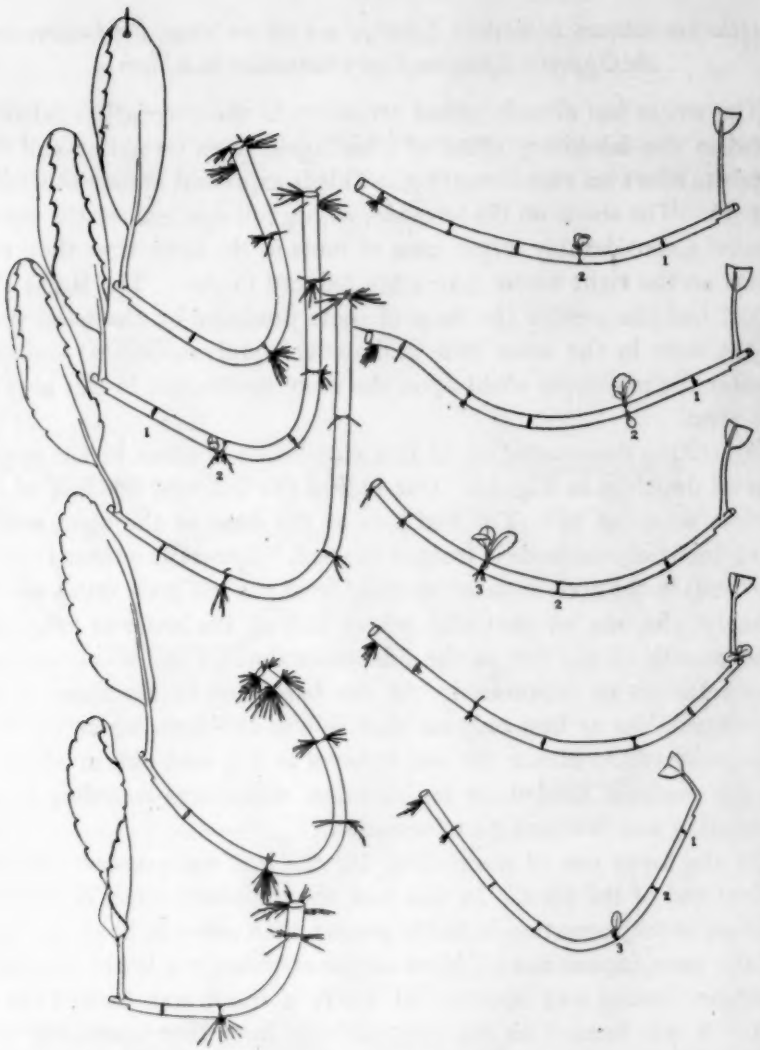

FIG. 15. Showing that reduction in size of apical leaf diminishes its inhibitory power. On the left, five stems each with large size leaf at apex, on upper side of stem. Inhibition of shoot formation complete except in one stem where a shoot is formed in the lower bud of the second node. On the right, five stems each with reduced leaf on the upper side. Four out of five stems form shoots and two of these do so in the upper bud of the second node. Root formation and geotropic curvature are considerably larger in stems with whole leaf than in stems with reduced leaf. Duration of experiment, Oct. 29 to Nov. 23.

Correlation between Inhibitory Effect of a Leaf on Shoot Production and the Opposite Effect on Root Production in a Stem.

The writer has already called attention to the correlation existing between the inhibitory effect of a leaf upon shoot formation and the opposite effect on root formation. This is expressed among others in Fig. 15. The stems on the left side, with a full size leaf at the apex, formed a considerably larger mass of roots in the same time than the stems on the right whose leaves are reduced in size. The larger the apical leaf the greater the mass of roots produced by the basal part of the stem in the same time and under equal conditions; and the greater the inhibitory effect upon the shoot production in this part of the stem.

A striking demonstration of this correlation is given in the upper row of drawings in Fig. 16. One-half of the leaf and one-half of its petiole were cut off. The leaf was at the base of the stem which contained only one node in front of the leaf. Generally only one of the two buds in the node situated apically from the leaf grew into a shoot; namely, the one on that side where half of the leaf was removed. The growth of the bud on the side where the half leaf was preserved was retarded or suppressed. At the basal end of the stems roots developed, but at first only on that side of the stem where the leaf was preserved.[4] Hence the leaf behaves as if it sent out, in addition to the material needed for regeneration, substances retarding shoot formation and favoring root formation.

In the lower row of stems (Fig. 16) the leaf was preserved at the apical end of the stem. In this case the inhibitory effect of the half leaf on shoot formation is much greater than when it is at the base of the stem (upper row). Most of the stems in the lower row have not yet formed any shoots, but where a shoot was formed (as in III*a*) it was formed on the opposite side from that where the half leaf was preserved, while the roots were on the same side with the leaf.

In Fig. 16 the half leaf was above. In Fig. 17 the half leaf was always below. The result in Fig. 17 is the same as in Fig. 16, *i.e.*, on the side where the half leaf is preserved the shoot formation is always retarded compared with that on the other side. The inhibition is more complete when the leaf is at the apex (lower row) than when the leaf is at the base (upper row).

[4] Later on roots may form on both sides of the basal end of the stem.

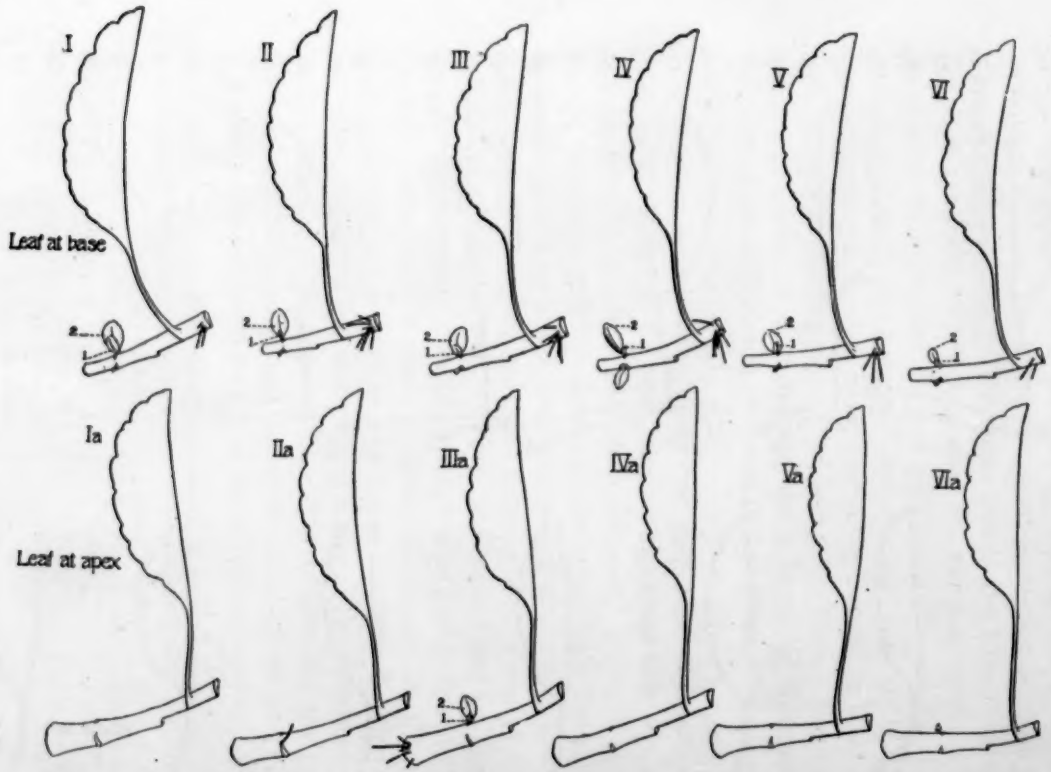

FIG. 16. Upper row: half leaf at base of stem. Shoot formation inhibited and root formation favored on that side of stem where petiole of leaf is preserved. Lower row: half leaf at apex. Inhibitory effect of leaf on shoot formation is more complete than in upper row. Duration of experiment, Oct. 24 to Nov. 9.

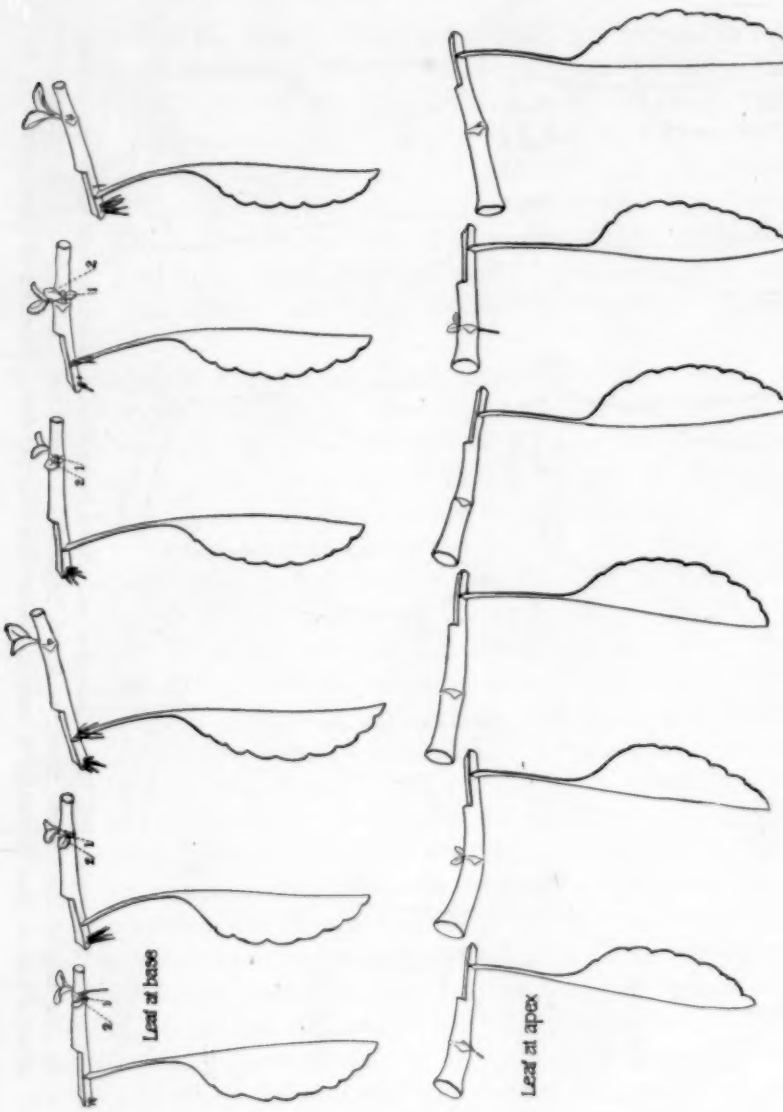

FIG. 17. Half leaf on lower side of stem. Similar result as in Fig. 16. Duration of experiment, Oct. 19 to Nov. 1.

Proof That the Leaf Sends Nutritive Material Also in the Basal Direction of the Stem.

When we suspend stems horizontally with one leaf at the apex, the inhibitory effect of the leaf upon shoot formation is much stronger when the apical leaf is on the upper side of the stem than when the leaf is on the lower side of the stem. We can use experiments of the latter type to show that the leaf sends nutritive substances to the base as well as to the apex and that the fact that the leaf inhibits shoot formation at the base is not due to the leaf failing to send nutritive material in the direction of the base of the stem. The method of proving this consists in measuring the influence of the mass of the leaf upon shoot formation in the basal part of a horizontally suspended stem.

Stems were split longitudinally and suspended horizontally; each stem having a leaf at the apex, and on the lower side of the stem.[5] Fig. 18 gives the result of such an experiment. Pieces of stems possessing two nodes and two leaves at the apical node are split longitudinally, so that each half stem has one leaf at the apex and one bud in the basal node. One leaf is left intact while the size of the sister leaf is reduced considerably. In Fig. 18 Leaves I and I*a*, II and II*a*, etc., are sister leaves. Practically each stem has produced a shoot at the basal node, but the shoot is invariably greater in the stems in the upper row where a whole leaf was at the apex than in the lower row where the apical leaf was reduced in size. The drawing was made on the 34th day of the experiment. It is obvious that the growth of the basal shoots increases with the mass of the apical leaves and this is proved by the relative weight of the leaves and shoots.

Duration of Experiment, 39 Days.

	Wt. of 4 whole leaves.	Mass of 4 shoots.	Shoots per gm. of leaf.
	gm.	*gm.*	*mg.*
Fresh.	17.035	2.128	125
Dry.	1.196	0.193	160
	Wt. of 4 reduced sister leaves.		
Fresh.	2.182	0.384	175
Dry.	0.140	0.027	193

[5] If the leaf is on the upper side, we get too much inhibition of shoot formation, whereby the experiment is rendered difficult.

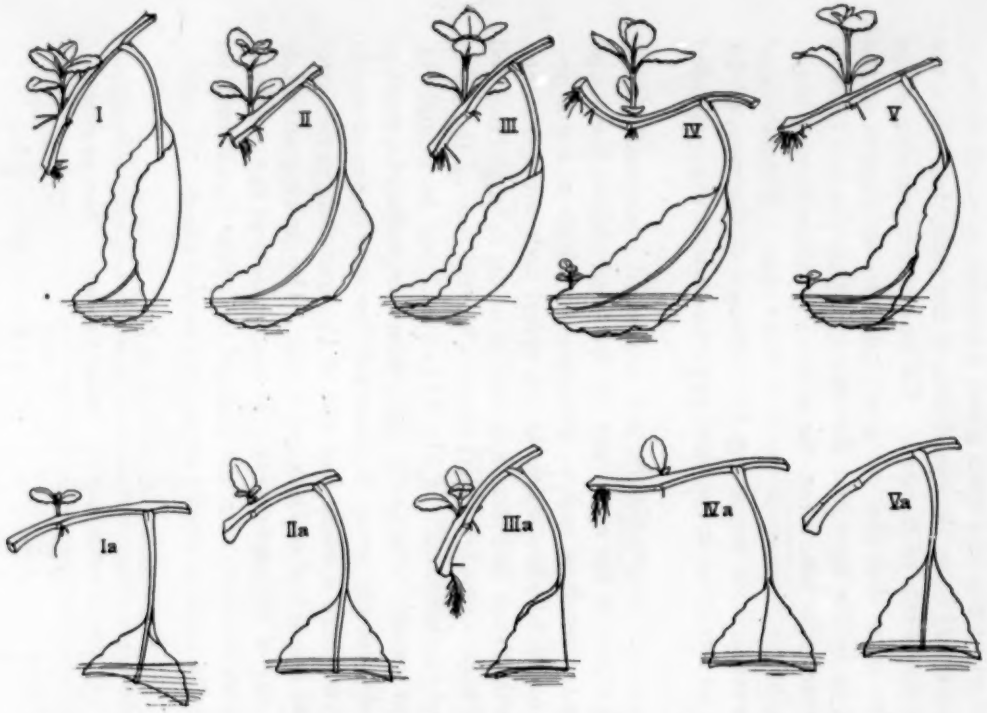

FIG. 18. Stems split longitudinally and suspended horizontally, with apical leaf on the lower side. Leaves I and I*a*, II and II*a*, etc., are sister leaves. The leaf in the upper row is intact, while in the lower row it is reduced in size. The mass of shoots produced is smaller in the stems of the lower row where the size of the leaf is reduced than in the upper row where the leaf is larger. This proves that inhibitory influence of leaf upon basal parts of stem is not due to lack of nutritive material.

Other experiments gave similar results. While the experiment does not reveal any strict proportionality between mass of leaf and mass of basal shoot produced, the increase of the mass of shoots with the mass of the leaves is unmistakable.

We therefore come to the following conclusion. The leaf sends material for growth in both directions of the stem, to the apex as well as to the base. It also behaves as if it sent out inhibitory substances in both directions, but if this be the case there must exist a considerable difference in regard to the mass of the latter. While much or almost all of the inhibitory substance is sent in basal direction, only traces of it are sent in an apical direction, so that special experiments are required to demonstrate the inhibitory effect in the apical parts of the stem.

A glance at Fig. 18 confirms also the statement that the mass of roots formed in the basal part of a stem increases with the mass of the apical leaf. The stems in the upper row with a whole leaf at the apex have a greater mass of shoots and roots than the stems in the lower row with a reduced leaf.

DISCUSSION.

We have shown in this paper that a leaf inhibits the regeneration of shoots in the basal parts of the stem and that this inhibition is diminished or ceases when the mass of the leaf is reduced below a certain limit. If the inhibitory influence of the leaf is due to inhibitory substances sent out by the leaf to the basal parts of the stem we must conclude that traces of these inhibitory substances flow also to the apex of the leaf since it is possible to demonstrate slight inhibitory influences of the leaf in the buds situated apically.

The influence of the leaf upon the regeneration of roots is exactly the reverse from that on the regeneration of shoots. The leaf favors the formation of roots in the basal parts of the stem and this favorable influence upon regeneration of roots in the basal part of a stem increases with the mass of the apical leaf.

This gives us an indication of the rôle which a leaf plays in the establishment of the polar character of regeneration in the stem of *Bryophyllum calycinum*. When a piece of stem is cut out with a leaf in the middle, the leaf sends out nutritive material in both directions of

the stem, since it can be shown that if once a shoot is caused to grow, it increases with the mass of the leaf, no matter whether the shoot is situated at the base or the apex. The leaf has a powerful inhibitory effect upon the development of basal shoots. If we assume this inhibitory influence to be due to inhibitory substances we must further assume that not more than mere traces of these inhibitory substances reach the apex which are not sufficient to interfere with the growth of shoots. At the moment we cut out the piece of stem from a plant the stem contains throughout a sufficient quantity of these inhibitory substances to prevent shoots from growing, and these inhibitory substances will continue to flow in the descending sap towards the base of the stem. The most apical buds in the stem will hence be the first ones to become sufficiently free from inhibitory substances to be able to grow and the regeneration of shoots will start at the apex of the piece of stem. As soon as the shoots are beginning to grow at the apex they in turn act like a leaf so that now the further growth of shoots at the base is permanently inhibited. On the other hand, the influences which inhibit shoot formation at the base are associated or identical with influences favoring root production. Hence the leaf will favor root formation at the base of the stem and shoot formation at the apex. This gives an idea how the leaf may contribute by its "internal secretion" to the establishment of the polar character of regeneration.

If it could be shown that plants possess a closed circulatory system comparable to that of animals, all these facts might become easily intelligible if we assume that inhibitory substances for shoot formation (and favorable substances for root formation) are carried in the descending sap from the leaf to the root, where they are retained or altered, so that the ascending sap becomes practically (but not absolutely) free from these substances and contains only the nutritive material for the formation of shoots.

The assumption that the inhibitory influence of a leaf upon shoot formation in the basal part of a stem is due to inhibitory substances, is not without analogy in biology. It is known that when twins in cattle have different sex the female is in the majority of cases sterile, and Lillie[6] has shown that there exists an exchange of blood between

[6] Lillie, F. R., *J. Exp. Zool.*, 1917, xxiii, 371.

such embryos. This indicates that there exists in the blood of the male cattle embryo an inhibitory substance which prevents the normal development of the sex glands of the female embryo.

A second case is that of the prevention of the development of the male plumage in the female fowl. Boring and Pearl[7] have shown that the ovary of such females contains specific cells, the lutear cells, which are absent in the male. Boring and Morgan[8] have found that in the Sebright, where the male shows hen-feathering, lutear cells exist in the testes of the male bird. Since extirpation of the ovary in fowl and duck leads to the assumption of the full male plumage by the female (Goodale[9]), it seems as if some specific substance in the ovary inhibited the development of male plumage in the female. This inhibitory substance may be contained in the lutear cells, which, however, cannot well influence the development of feathers in any other way than by the secretion of some substance into the blood. The assumption that the inhibition of shoot formation in the basal part of a stem by a leaf is due to an inhibitory substance secreted by the leaf is therefore not without a precedent.

It is, however, necessary to call attention to the fact that even if the inhibitory influence of the leaf upon shoot formation should turn out to be based on the chemical character of the sap sent out by the leaf, it does not follow that all phenomena of inhibition and correlation in regeneration will find their explanation on the same basis. Quantitative experiments published in a former paper suggest that the inhibitory influence of a piece of stem on shoot formation in the leaf of *Bryophyllum calycinum* is due to the fact that the leaf sends its sap normally to the stem, and that as long as this happens the buds in the notches of the leaf cannot grow out.[10]

We shall show in the next communication that growing buds have inhibitory influences upon the formation of shoots comparable to the same influences caused by a leaf.

[7] Boring, A. M., and Pearl, R., *Anat. Rec.*, 1917, xiii, 253. Pearl and Boring, *Am. J. Anat.*, 1918, xxiii, 1.

[8] Boring, A. M., and Morgan, T. H., *J. Gen. Physiol.*, 1918, i, 127.

[9] Goodale, H. D., *Biol. Bull.*, 1910–11, xx, 35; *Am. Nat.*, 1913, xlvii, 159; *J. Exp. Zool.*, 1916, xx, 421.

[10] Loeb, *Ann. Inst. Pasteur*, 1918, xxxii, 1.

SUMMARY.

1. In *Bryophyllum calycinum* two apical leaves suppress the shoot formation in all the dormant buds situated basally from the leaf; one apical leaf suppresses the shoot formation in the basal buds situated in the same half of the stem where the leaf is, and, if one-half of the petiole of such a leaf is removed, the growth of basal buds in one quadrant of the stem is suppressed.

2. This inhibitory influence of a leaf upon shoot formation in the basal part of a stem is diminished or disappears when the mass of the leaf is reduced below a certain limit.

3. The inhibitory influence of an apical leaf upon the growth of shoots in horizontally suspended stems is greater when the leaf is on the upper than when it is on the lower side of the stem.

4. All these facts suggest the possibility that the inhibitory influence of the leaf upon shoot formation is due to inhibitory substances secreted by the leaf and carried by the sap from the leaf towards the base of the stem.

5. An apical leaf accelerates root formation in the basal part of a stem and this accelerating effect increases with the mass of the leaf.

6. This inhibitory influence of a leaf upon shoot formation and the favoring influence upon root formation in the more basally situated parts of the stem is one of the factors determining the polar character of regeneration.

AMPHOTERIC COLLOIDS.

III. Chemical Basis of the Influence of Acid upon the Physical Properties of Gelatin.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 27, 1918.)

I.

Many of the authors who have worked on the physical chemistry of proteins, like Hardy, Pauli, Michaelis, Robertson,[1] and others, have pointed out that the different properties of proteins, *e.g.* swelling, viscosity, are affected by electrolytes in a parallel way, a fact which suggests that these variations are due to the same variable. The nature of this variable is not known and the majority of authors believe it to be connected with the colloidal character of the proteins, while others are inclined to assume a purely chemical or stoichiometrical relation. The reason for this doubt lies in the fact stated appropriately by Pauli[2] in discussing the influence of acid and alkali upon the osmotic pressure of gelatin.

> Pauli and Handowski have pointed out that in these experiments too the essential feature is the formation of ionic protein. But a satisfactory explanation of this increase is still lacking, because we have no measurements of the molecular concentrations with the aid of other methods, which prove that we are dealing with a true osmotic pressure in the sense of van't Hoff.

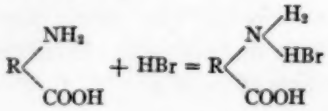
Pauli assumes that the ionized protein undergoes a stronger "hydratation" than non-ionized protein and that this hydratation explains the swelling of gelatin, as well as the apparent osmotic pressure, the latter being only a phenomenon similar to swelling.

[1] The reader is referred for the literature on the subject to Robertson, T. B., The physical chemistry of the proteins, New York, 1918.

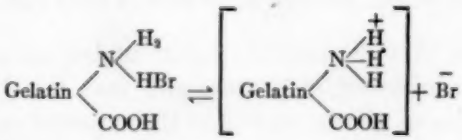
[2] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 245.

It seemed to the writer that experiments on gelatin might permit us to satisfy the demand of Pauli; namely, to supply the molecular measurements necessary to show that the osmotic pressure and the other properties of gelatin solutions vary in proportion to the amount of acid combining with a given amount of gelatin. In two previous papers the writer has already shown that this is true for the influence of neutral salts on these properties of gelatin.[3] Procter's[4] experiments also indicate a purely stoichiometric basis for the influence of acids on the swelling of gelatin.

According to Werner[5] amphoteric electrolytes are characterized by their ability to add H ions or OH ions and not by their ability to give off H and OH ions. It is generally assumed, and probably correctly, that when an acid like HBr combines with an amino-acid or a protein, the reaction occurs in an NH_2 group of the amino-acid or protein. According to Werner when NH_3 and HBr combine, the positively charged hydrogen ion of HBr is attached by a secondary valency to the N whose three negative charges now hold four positively charged H ions. No other positive ion except H can act in this way. The Br is able to dissociate as freely in the NH_4Br as in the free acid. The same assumption is to be made for the way an acid, *e.g.* HBr, combines with amino-acids or proteins.



Such a molecule, *e.g.* gelatin bromide, dissociates into a positively charged gelatin ion and a negatively charged Br ion, the H ion of the HBr now forming a part of the complex and positively charged gelatin ion.



[3] Loeb, J., *J. Gen. Physiol.*, 1918, i, 39, 237.

[4] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307. Procter, H. R., and Burton, D., *J. Soc. Chem. Ind.*, 1916, xxxv.

[5] Werner, A., Neuere Anschauungen auf dem Gebiete der anorganischen Chemie, Braunschweig, 2nd edition, 1909.

Such a gelatin salt can only exchange the Br with the anion of a neutral salt and it is impossible for a complete molecule of a neutral salt like KCl to combine with the NH_2 group as has been assumed. The writer's experiments on the action of neutral salts on gelatin treated previously with acid are in harmony with the ideas of Werner and opposed to the assumption of a pentavalent N atom in the protein molecule capable of adding a whole molecule of a neutral salt. We do not know yet whether only one or more NH_2 groups in the gelatin molecule are able to bind a molecule of HBr.

Gelatin is an amphoteric electrolyte which at the isoelectric point (which for gelatin lies at pH = 4.7) is practically insoluble. When we prepare a gelatin solution and give it a hydrogen ion concentration of 2.10^{-5} (*i.e.* pH = 4.7), the solution in less than 24 hours becomes opaque on cooling in as low a concentration as 0.25 per cent and probably at any concentration; except that the opacity due to the insolubility becomes too slight in very low concentrations to be noticeable. This explains why gelatin at the isoelectric point has practically no osmotic pressure, no swelling, a minimal conductivity, viscosity, etc.

When we treat isoelectric gelatin with a limited quantity of HBr of a low concentration a certain amount of gelatin is transformed into gelatin bromide, which is soluble and dissociates electrolytically. The higher the concentration of acid used the more gelatin bromide is formed and the more molecules of gelatin go into solution, until at a certain point all the insoluble gelatin molecules are converted into soluble gelatin bromide molecules. Since a 1 per cent gelatin bromide solution should possess the same degree of electrolytic dissociation as the HBr combined with it and since a 1 per cent gelatin bromide solution on account of the high molecular weight of gelatin must be considered as a very dilute solution, we shall commit no great error in assuming a complete electrolytic dissociation of the gelatin bromide. If it is true that the increase in osmotic pressure of gelatin under the influence of HBr is merely due to an increase in the number of soluble gelatin molecules, it must be possible to show that the osmotic pressure in this case increases approximately with the number of gelatin bromide molecules formed. This we intend to prove in the present paper.

While Pauli is right in stating that gelatin treated by acid is more highly ionized than common gelatin, I cannot agree with him that it is this ionization which causes the difference in the osmotic pressure of the gelatin at the isoelectric point and gelatin treated with acid. The increase in osmotic pressure is as our numerical results will show merely the expression of the increase in the number of particles in solution and there is no need or room for the assumption that the hydratation or any other quality except the number of particles in solution has anything to do with this increase in pressure.

Since viscosity and swelling vary practically parallel with the osmotic pressure, these phenomena must also be a function of the number of particles or ions in solution.

Hardy[6] has pointed out that the ionization of a protein increases its viscosity, and the increase of viscosity of gelatin with its ionization might then account for the parallelism between the curves for the bromine number and for the viscosity of the gelatin solution.

As far as a theory of swelling is concerned, the only one possessing any quantitative basis at present is Procter's.[4]

If we can, therefore, prove that under the influence of HBr the osmotic pressure of gelatin changes in proportion with the gelatin bromide formed, we have no further reason to question the purely chemical or stoichiometrical basis of the influence of acid upon all the physical properties of gelatin.

II. Measurements without Washing.

1 gm. of finely powdered gelatin is put for 30 minutes at 15°C. into each of a series of beakers containing 100 cc. of HBr of a different concentration, varying from M/8 to M/8192. As a control 1 gm. of gelatin is put for 30 minutes at 15°C. into 100 cc. of distilled water. The powdered gelatin is then poured into a cylindrical funnel and the acid allowed to drain off. The diameter of all the funnels being the same, the height of the gelatin gives a measure for the relative amount of swelling. Each gram of gelatin is then put into 100 cc. of HBr of the same concentration with which it had been treated before and is liquefied by heating to 50°C. Immediately after melting the time

[6] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.

of outflow through an Ostwald viscometer is ascertained at a temperature of 24°C. By keeping temperature and time required for melting and time between completing this process and the viscosity measurement constant in each case comparable results are obtained.[7] The time of outflow of distilled water through the viscometer was 55 seconds. The two curves of Fig. 1 give the values for swelling and viscosity, with the logarithms of the concentration of acid used as abscissæ. Under each acid is found the pH for the gelatin solution ascertained after the viscosity determination.

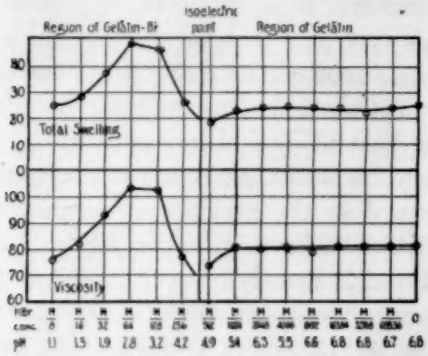


FIG. 1. Curves for viscosity and swelling of gelatin in the presence of various concentrations of HBr. Abscissæ, logarithms of concentration of HBr used. Under each concentration is found the pH of the solution. Isoelectric point and minimal values for curves at pH about 4.7. Both curves parallel, showing a maximum at pH between 2.8 and 3.2, and a drop when pH < 2.8.

The rest of the liquefied solution of gelatin in acid was then put into bags of collodion,[8] to ascertain the osmotic pressure of the gelatin solution, the pressure being expressed in mm. of height of the column of 1 per cent gelatin solution in the glass tube inserted through the tightly fitting rubber stopper closing the bag of collodion. The

[7] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 395.

[8] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127. Loeb, *J. Biol. Chem.*, 1918, xxxv, 497. It is hardly necessary to state that these bags are freely permeable for HBr and that HBr produces no osmotic pressure when put in such bags. There is a slight rise of the column of liquid in the manometer at the beginning of the experiment which disappears completely in a few hours, while the rise due to the gelatin, for which the membrane is impermeable, is permanent.

outside liquid was in each case HBr of the same concentration as that in which the gelatin was dissolved. The whole experiment was carried on in a water bath of a temperature of 24°C. The curve at the top in Fig. 2 is the curve for the osmotic pressure observed after about 20 hours, at which time equilibrium was established.

The conductivity of the gelatin solution was then ascertained, as well as the amount of Br found in 25 cc. of 1 per cent gelatin solution. The curves are given in Fig. 2. A glance at Fig. 2 will show that the curve for osmotic pressure is *not* parallel to the curves for conductivity and for the Br number. It is, therefore, impossible to arrive from experiments of this type at a decision whether the influence of HBr (or other electrolytes) upon gelatin is of a stoichiometrical or of a colloidal character. Yet those familiar with the literature of this subject will remember that the conclusions of most colloid chemists are based on experiments in which the action of the electrolyte upon the protein was measured in the presence of an excess of electrolyte. The second fact which deserves attention becomes clear by a comparison of Figs. 1 and 2; namely, that the minima which appear in the two sets of curves lie at different acid concentrations: in Fig. 1 between M/256 and M/512, in Fig. 2 (for osmotic pressure) between M/2048 and M/4096. Comparing, however, the pH in the two sets of curves we notice that the minimum is at the same pH, namely about 4.7, which is the isoelectric point for gelatin. A good deal of the work on which the colloidal theory of the behavior of proteins rests was done without any measurements of pH and by plotting the effect against the concentration of the acid. It is no wonder that work with two such shortcomings in its method did not furnish any proof for the stoichiometrical character of the action of electrolytes on the physical properties of amphoteric colloids.

III. Effect of Washing.

Our method consists in removing the excess of HBr (or of any other electrolyte) after it has had a chance to act on the gelatin. 1 gm. of powdered gelatin is put again into each of a series of beakers for 30 minutes at about 15° or 20°C., each beaker containing 100 cc. of HBr of a different concentration. Then the gelatin is poured on a

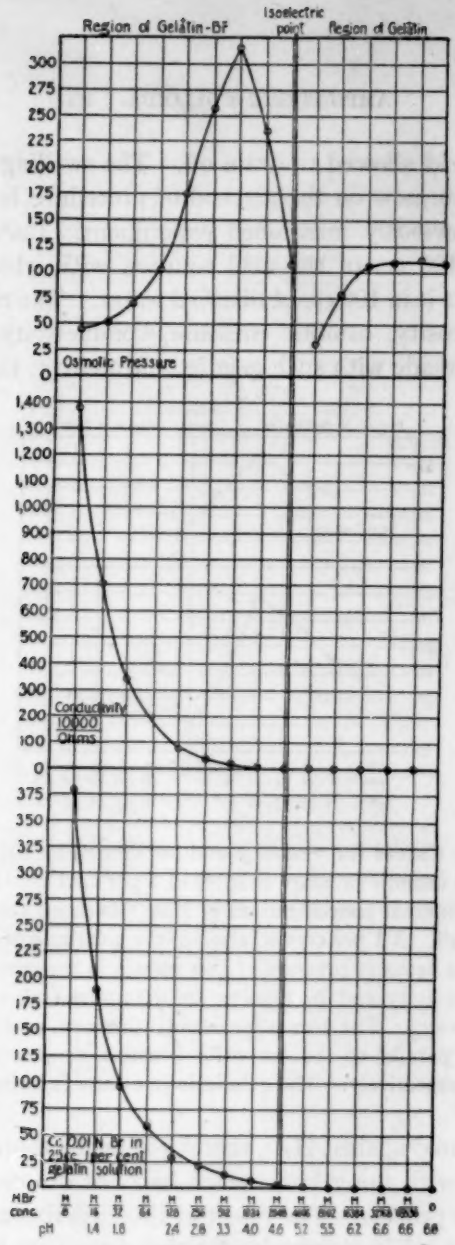
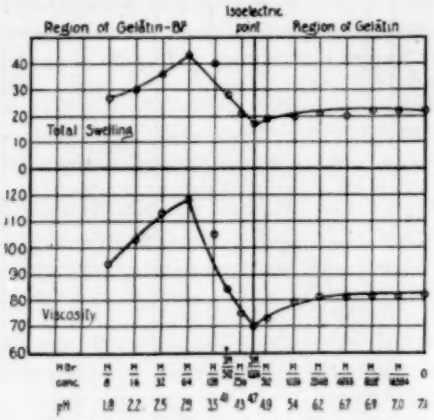


FIG. 2. Curves for bromine number (cc. 0.01 N Br in 25 cc. of 1 per cent gelatin solution), conductivity, and osmotic pressure of gelatin in presence of various concentrations of HBr. Abscissæ as in Fig. 1. No parallelism between curve for osmotic pressure and curves for bromine number and conductivity. Curve for osmotic pressure has minimum at pH about 4.6, maximum at pH = 3.3, and then drops. No conclusion can be drawn from such experiments as to the "colloidal" or true character of osmotic pressure. Osmotic pressure measured in height of column of 1 per cent gelatin solution.

filter and the acid allowed to drain off. The swelling is measured as described. From now on the method of procedure is different from that in the previously mentioned experiment. Instead of melting the gelatin in 100 cc. of the acid solution with which it had been treated, we melt it in 100 cc. of distilled water. The rest of the determinations—viscosity, osmotic pressure, conductivity, and titration for Br—are all made with such gelatin. Moreover, the osmotic pres-



FIGS. 3 and 4. Curves for viscosity and swelling (Fig. 3); bromine number, conductivity, and osmotic pressure (Fig. 4) of 1 per cent gelatin solution treated previously with different concentrations of HBr (abscissæ) the acid having been allowed to drain off. A 1 per cent solution of the gelatin *in distilled water* is then prepared, and the osmotic pressure of this gelatin is measured against distilled water, and conductivity and Br number are determined after 20 hours dialysis against distilled water. The curves for osmotic pressure, swelling, viscosity, and conductivity are parallel to the curve for bromine number from pH = 4.7 to pH = 2.9 or 3.3 respectively. The gelatin is free from bromine for pH $\lesssim$ 4.7.

sure was measured against H_2O, thereby allowing more of the free acid not combined with the gelatin which had not drained off to diffuse out during the process. The result of this experiment is represented in Figs. 3 and 4. Fig. 4 contains the measurements for osmotic pressure and Br number, and the curves are almost parallel (with the exception of the value for the osmotic pressure for gelatin treated with M/8 acid). This parallelism is the missing link which allows us to decide in favor of the purely chemical and against the colloidal conception

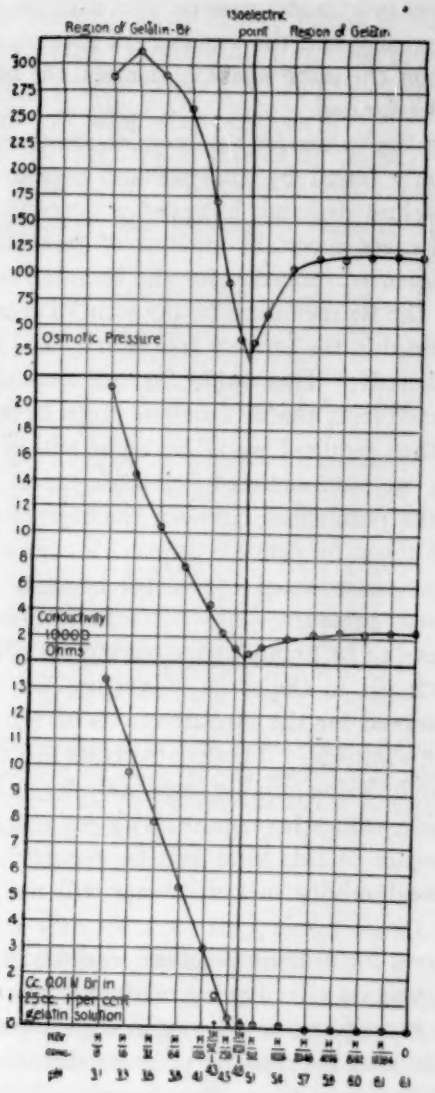


FIG. 4. See explanation under Fig. 3.

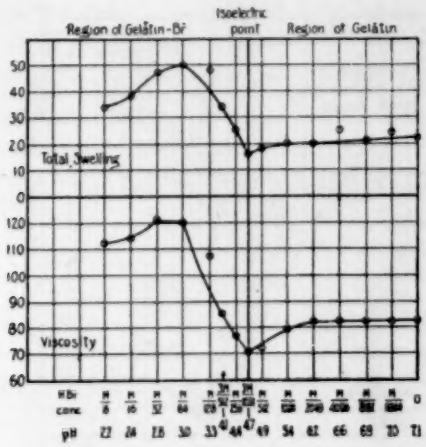
of the influence of electrolytes upon the osmotic pressure. Since the bromine number of gelatin increases parallel with the osmotic pressure (and also the viscosity and the swelling) of gelatin, these properties must depend upon the same variable; namely, the number of gelatin bromide molecules formed.

We are giving the values for the bromine number and for conductivities as actually found by analysis and measurements of resistance. These values demand, however, a correction owing to the fact that in all cases a certain amount of free HBr was present. The actually measured quantity for the bromine number is in each case the sum of the Br contained in the form of gelatin bromide and of the Br contained in the form of free HBr. The latter can be calculated from the pH. This value for the quantity of free HBr should be deducted from the Br numbers given in the curves. Since the correction thus required would be slight within the limits of pH from 4.7 to 3.6, we have omitted it in this paper. We may state, however, that the parallelism between the corrected curves for the Br number and those for osmotic pressure is even more perfect than that between our uncorrected curves for bromine number and the curves for osmotic pressure. The correction necessary for the conductivity curves can be found by measuring the resistance of a HBr solution for each pH in the same measuring cell (with fixed electrodes) which served for the measurements of the resistance of the gelatin bromine solutions. These corrections are greater than those required for the bromine number, especially for $pH < 3.9$. In order to obtain reliable values for conductivity we must use purified gelatin. Experiments of this kind will be reported in a subsequent paper. The conductivity measurements will not be considered in this paper.

All three curves for osmotic pressure, viscosity, and bromine number show a drop again after having reached a maximum. This drop exists in a still more pronounced way in the curves for viscosity and swelling than in that for osmotic pressure, because the gelatin contained more HBr before than after dialysis. This drop is of great theoretical significance because it shows free HBr is present in excess of the binding capacity of gelatin for HBr. The free HBr represses the ionization of gelatin bromide on account of the common Br ion and

this causes the drop in the curves for the osmotic pressure of the gelatin, since the free HBr, being able to diffuse through the collodion membrane, cannot cause any increase in osmotic pressure. The drop begins usually when pH becomes <3.3 and the drop is the more considerable the more pH falls below this level.

The correctness of this view is proved by the fact that if we wash away the traces of free acid left in the capillary spaces between the particles of gelatin after the process of draining, by perfusing the



FIGS. 5 and 6. Same curves as in Figs. 3 and 4 except that the gelatin after the acid had been allowed to drain off was washed once with 25 cc. of H_2O. Parallelism between curves for Br number, conductivity, osmotic pressure (Fig. 6), viscosity, and swelling from pH = 4.7 to pH = 3.0. No Br found for pH $\lesssim$ 4.7.

gelatin on the filter with 25 cc. of H_2O, and if we allow the water to drain off also before we make up the gelatin into a 1 per cent solution in distilled water, the drop will disappear, as is obvious from Figs. 5 and 6. In Fig. 6 the drop has disappeared, the pH going only to 3.3, and in these curves there is as complete a parallelism between the bromine number and the osmotic pressure of the gelatin solution as the strict validity of the theory of van't Hoff demands. We still observe the drop for the curves for viscosity and swelling, but the pH in these cases falls below 3.3; namely, to 2.2 (Fig. 5).

If instead of giving one washing we give four washings with 25 cc. of H_2O after the acid has drained off, we avoid the low values of pH completely and the drop in the curves for swelling disappears (Fig.

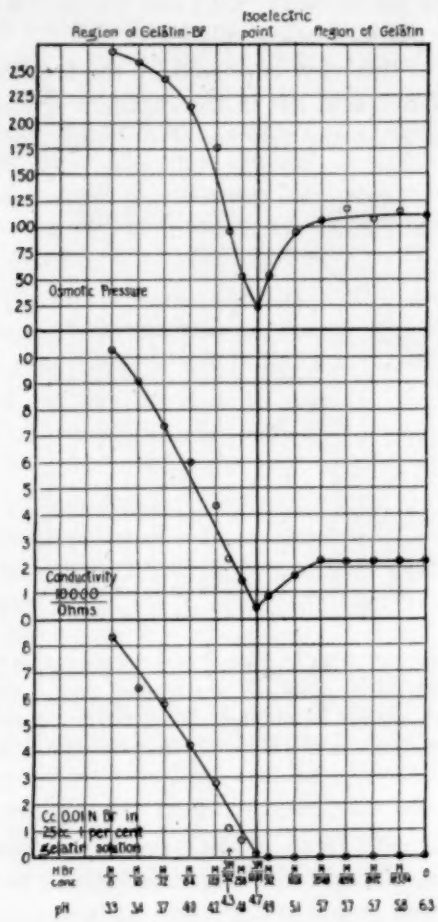


FIG. 6. See explanation under Fig. 5.

7). The parallelism between the curves for bromine number, for osmotic pressure and swelling is now practically complete.

The curves show distinctly that the independent variable is the bromine number. Thus in Fig. 7 this number was slightly in ex-

cess in the gelatin treated with M/128 and M/64 HBr. Corresponding abnormal values are found in the curves for conductivity, osmotic pressure, and swelling. The same is shown in Fig. 8. It

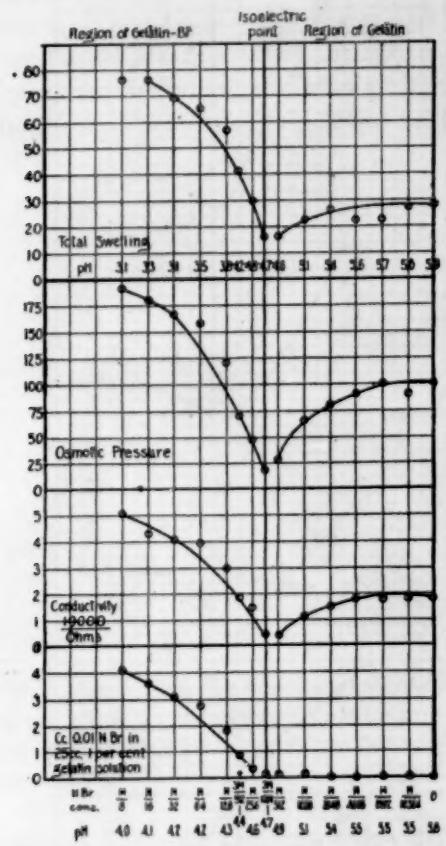


FIG. 7. Same curves as in preceding figures except that gelatin, after the acid treatment, had been washed four times with H_2O. Explanation as in preceding figures. Notice that curves are parallel and the gelatin is free from Br for pH $\lesssim$ 4.7.

is, therefore, the Br number which determines the curves, *i.e.* the amount of gelatin bromide formed. Fig. 8 gives the curves for eight washings. Again the parallelism between the curves for the Br number and the other physical properties is obvious.

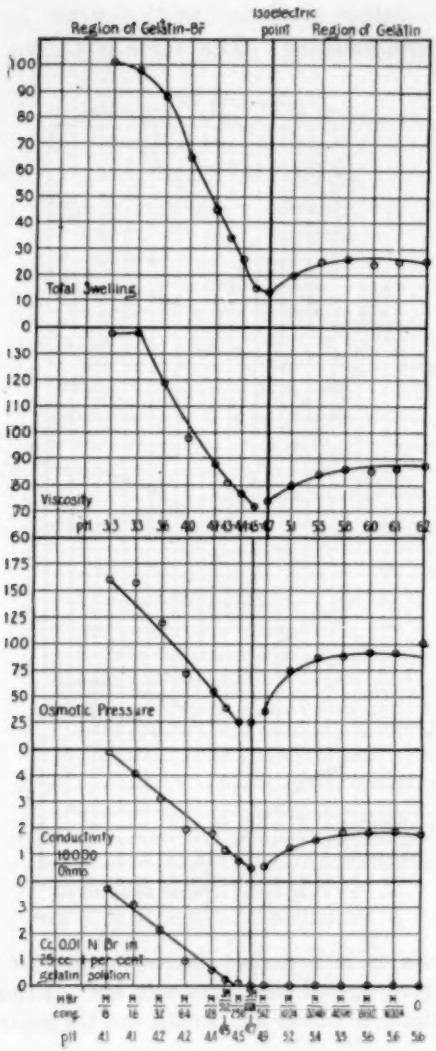


FIG. 8. Same curves as in preceding figures except that gelatin had been washed eight times with H_2O. Curves parallel and gelatin free from Br for pH $\lesssim$ 4.7.

These experiments furnish the proof that the effect of acid (HBr) upon the physical properties of gelatin is the unequivocal function of the amount of gelatin bromide formed; the probable reason for this being that pure gelatin (as it exists at the isoelectric point) is practically insoluble (and undissociated), while gelatin bromide is soluble.

We must now furnish the proof that not only is there a parallelism between the curve for the bromine number on the one hand and the curves for the physical properties of gelatin treated with HBr, but

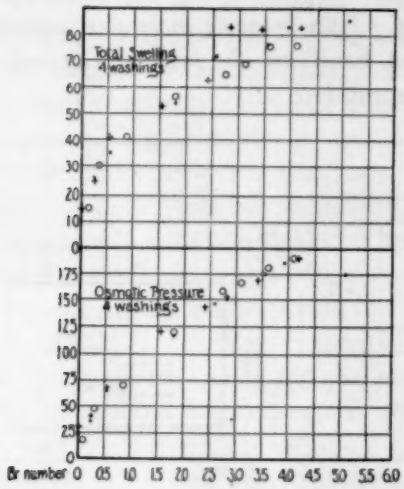


FIG. 9. The abscissæ represent the bromine number, the ordinates the values for osmotic pressure found in three different experiments with 1 per cent gelatin solution previously treated with HBr and washed four times with water. Notice agreement between values.

that to each definite Br number belongs a definite and absolute value for osmotic pressure, conductivity, swelling, and viscosity. We can do this by plotting the results of different experiments with the values for Br numbers as abscissæ and the values for osmotic pressure, swelling, etc., as ordinates. In this case the values obtained for osmotic pressure in the different experiments should differ only within the limits of the accuracy of our measurements.

In Fig. 9 the curves for three different experiments with four washings each are plotted for osmotic pressure and for swelling. The

variations lie within the limit of error. In the experiments plotted in Fig. 10 the number of washings of gelatin varied. In spite of the difference in the treatment we notice that for the same Br number practically the same value of osmotic pressure was found in all experiments. Since the curves for the other properties are parallel to the curve for osmotic pressure, it is not necessary to reproduce all the curves.

We therefore reach the conclusion that the variation of the physical properties of gelatin under the influence of HBr is an unequivocal function of the number of gelatin bromide molecules formed and that colloidal speculations not based on the laws of classical chemistry are neither needed nor warranted.

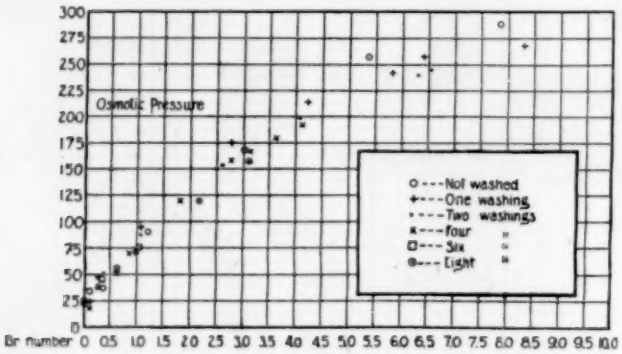

Osmotic Pressure
Not washed
One washing
Two washings
four
Six
Eight
Br number 0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0

FIG. 10. Abscissæ represent bromine number, ordinates, values for osmotic pressure found in different experiments with 1 per cent gelatin solution previously treated with HBr and washed a different number of times.

IV. Titration of Gelatin with NaOH.

The following facts constitute an important link in the proof for a chemical theory of the action of electrolytes (in our case HBr) upon the physical properties of gelatin.

We notice that in all the curves the gelatin was found to be absolutely free from Br at the isoelectric point as well as on the alkaline side from the isoelectric point; *i.e.*, it was free from Br whenever pH $\overline{>}$ 4.7. Yet this gelatin had been treated with HBr. The fact that HBr cannot combine or remain in combination with gelatin for

pH $\overline{>}$ 4.7 is a further proof of the correctness of the purely chemical theory according to which for pH > 4.7 ionized gelatin can only exist as an anion, not capable of holding HBr in combination.

When we titrate 25 cc. of 1 per cent gelatin solution at the isoelectric point with 0.01 N NaOH, we find that it acts as an acid, requiring between 5.25 and 5.5 cc. 0.01 N NaOH for neutralization against phenolphthalein. (The number of cc. 0.01 N NaOH required to neutralize 25 cc. of 1 per cent gelatin we will call the NaOH number of gelatin.) When the pH of common gelatin, not treated with acid, is greater than 4.7 the NaOH number becomes less than 5.25 cc., probably on account of the fact that part of the gelatin exists as a metal gelatinate (probably chiefly Ca gelatinate) owing to ionogenic impurities remaining from the process of manufacture. Our previous papers have shown that at the isoelectric point gelatin is compelled to give off these ionogenic impurities.

On the more acid side from the isoelectric point the gelatin contains Br and the Br number increases with decreasing pH. With the exception of a small fraction this Br is held in combination with the gelatin as can be shown on the basis of titration with NaOH of the gelatin treated previously with HBr and possessing a pH < 4.7. In such a titration the gelatin solution whose pH < 4.7 is gradually rendered more alkaline through the addition of NaOH until finally its pH becomes equal to 4.7, and when that happens all the HBr held in combination with gelatin must be set free. As a consequence in a titration of gelatin bromide with NaOH two acids must be saturated with NaOH, the pure gelatin, and the HBr set free when during the process of titration the gelatin reaches its isoelectric point. It follows from this that the NaOH number found in this case must equal the sum of the Br number of the gelatin plus the NaOH number for gelatin at the isoelectric point; regardless of how the gelatin had been treated before and regardless of the pH for which this rule is tried out.

If we denote a given pH with n, the NaOH number at this pH = n as "$(NaOH)_n$," the NaOH number at the isoelectric point with "NaOH (isoelectric)," and the Br number at pH = n with "Br_n", then the following equation will hold: $(NaOH)_n$ = NaOH (isoelectric) + Br_n. In Table I, I have selected at random four experiments in which the

NaOH number and the Br number for different values of pH are given. It is easy to show that the equation holds within the limits of accuracy of our experiments. Thus the NaOH number for the isoelectric point is practically a constant in all experiments, namely between 5.25 and 5.5; and this value represents the binding capacity of "pure" gelatin for NaOH; or in other words, the binding capacity of 0.25 gm. of gelatin freed from ionogenic impurities is between 5.25 and 5.5 cc. 0.01 N NaOH, with phenolphthalein as indicator. Thus

TABLE I.

Experiment I. No washings, but made up in H_2O and dialyzed against H_2O.

pH	3.1	3.3	3.6	3.8	4.1	4.3	4.5	*4.8*	5.1	5.4	5.7	5.8	6.0	6.1
Br number.	13.35	9.75	7.85	5.35	3.0	1.2	0.35	*0.1*	0.1	0.1	0	0	0	0
NaOH "	18.00	15.00	13.50	11.50	9.0	8.0	7.00	*5.0*	4.0	2.5	1.75	1.5	1.5	1.5

Experiment II. One washing.

pH	3.3	3.4	3.7	4.0	4.2	4.3	4.4	*4.7*	4.9	5.1	5.7	5.7	5.8	6.3
Br number.	8.3	6.4	5.8	4.2	2.75	1.05	0.6	*0.1*	0	0	0	0	0	0
NaOH "	12.5	12.0	11.0	10.0	9.50	7.5	7.0	*5.5*	4.5	3.0	2.0	1.5	1.5	1.5

Experiment III. Four washings.

pH	3.8	4.0	4.1	4.2	4.3	4.5	*4.8*	5.0	5.3	5.6	5.7	5.8	5.9
Br number	4.15	3.45	2.85	2.4	0.55	0.25	*0*	0	0	0	0	0	0
NaOH "	9.00	8.50	8.00	8.0	7.0	6.00	*5.5*	4.0	2.5	2.25	1.5	1.5	1.5

Experiment IV. Eight washings.

pH	4.1	4.1	4.2	4.2	4.4	4.5	4.5	*4.7*	4.9	5.2	5.4	5.5	5.6
Br number	3.7	3.1	2.15	0.95	0.6	0.25	0.1	*0*	0	0	0	0	0
NaOH "	8.0	8.0	7.0	6.5	6.5	6.00	5.5	*5.25*	4.5	3.25	2.5	2.5	2.0

in Experiment III, for pH = 4.1, $(NaOH)_n$ = 8.0, NaOH (isoelectric) = 5.5, and Br_n = 2.85. 5.5 + 2.85 = 8.35, while $(NaOH)_n$ actually found is 8.0. Table II gives a comparison of the agreement in all the experiments.

In Table II we call the sum of NaOH (isoelectric) + Br_n the calculated and $(NaOH)_n$ the observed value for a given pH. The table shows that the calculated and observed values agree within the limits of the degree of accuracy of the experiments.

We have now to make sure that the HBr measured in these titrations

CORRECTION.

On page 381, Vol. i, No. 3, January 20, 1919, line 11, for $pH > 4.7$ read $pH < 4.7$.

is practically the HBr which was in combination with the gelatin, and not free HBr left in the capillary spaces between the particles of powdered gelatin after the treatment of the latter with HBr. The reader will remember that in Experiment I (Table I) the acid was allowed to drain off and that the gelatin solution was afterwards dialyzed for about 20 hours against distilled water. In the other experiments, in addition to this the last traces of acid were removed by one or more additional washings with distilled water. It is a striking fact that for pH $\leqq$ 4.7 the gelatin was always free from Br although it had been treated with HBr. This coincidence of the point where Br begins to appear in the gelatin with the value pH $\gtrless$ 4.7 where it theo-

TABLE II.

Experiment I.			Experiment II (two washings).			Experiment III (four washings).			Experiment IV (eight washings).		
pH	$(NaOH)_n$		pH	$(NaOH)_n$		pH	$(NaOH)_n$		pH	$(NaOH)_n$	
	Calculated.	Found.		Calculated.	Found.		Calculated.	Found.		Calculated.	Found.
4.7	5.5		4.7	5.5		4.7	5.5		4.7	5.5	
4.5	5.85	7.0	4.4	6.1	7.0	4.5	5.75	6.0	4.5	5.35	5.5
4.3	6.7	8.0	4.2	8.25	9.5	4.2	7.9	8.0	4.5	5.5	6.0
4.1	8.5	9.0	4.0	9.7	10.0	4.1	8.3	8.0	4.4	5.85	6.5
3.8	10.8	11.5	3.7	11.3	11.0	4.0	9.0	8.5	4.2	6.2	6.5
3.6	13.3	13.5	3.4	11.9	12.0	3.8	9.6	9.0	4.2	7.4	7.0
3.3	15.2	15.0	3.3	13.8	12.5				4.1	8.3	8.0
3.1	18.8	18.0							4.1	8.9	8.0

retically should begin to appear speaks already against the assumption that the Br number is the expression of free HBr not drained or dialyzed or washed off.

The direct proof lies, however, in a comparison between the pH and the Br number. We select at random in Experiment III (Table I) pH = 4.0. The Br number found in 25 cc. of gelatin solution is for this pH 3.45 cc. 0.01 N Br. In the form of free acid this Br number would represent a hydrogen ion concentration of $\frac{3.45}{2,500}$ N, which is approximately $\frac{1}{700}$ N, while the actual normality of the gelatin solution was $\frac{N}{10,000}$; *i.e.*, less than one-tenth of $\frac{N}{700}$ (about 7 per cent). Hence more than 90 per cent of the HBr existed in chemical com-

bination with the gelatin and the small amount of free acid found was probably due to hydrolytic dissociation of gelatin bromide or to a trace of HBr not removed. This quantity of free acid is the correction of the value for the bromine number referred to in an earlier part of this paper. When, however, the free HBr is not washed off, as was the case in the experiment represented in Fig. 1, or when the free acid is only incompletely removed, the value $(NaOH)_n$ will represent, of course, more or less free acid and in this case the discrepancy between $(NaOH)_n$ and the pH found will be correspondingly smaller (see Fig. 1). This, however, manifests itself by the fact that a further rise in the Br number is no longer accompanied by a corresponding rise or is accompanied by a drop in the curves for osmotic pressure, swelling, and viscosity.

We may, therefore, consider it as proved that the bromine numbers given in this paper represent practically the HBr held in chemical combination by the gelatin with the exception of the small amount to be deducted owing to the presence of free HBr which can be calculated from the pH.

V. Theoretical Remarks.

Our experiments show that the influence of hydrobromic acid upon the physical properties of gelatin has a purely chemical or stoichiometrical basis. Gelatin and probably all proteins and amphoteric colloids behave as if they were merely amphoteric electrolytes capable of adding a H or OH ion.

Whether a protein adds an acid or a base depends on the hydrogen ion concentration; when the hydrogen ion concentration exceeds a critical point (which for gelatin is $C_H = 2.10^{-5}$), the gelatin will add acid; when it is lower it will add base. This critical hydrogen ion concentration is the isoelectric point. When gelatin is at the isoelectric point, it is free from ionogenic impurities and this "pure" gelatin is practically insoluble and hence can have no osmotic pressure, and, moreover, all the properties which depend upon its solubility are a minimum. When it is transformed into a salt by the addition of an acid (or a base) it becomes soluble, provided it is in combination with a monovalent ion, like Br or Na, etc. When pure gelatin is

transformed into a salt with monovalent anion or cation, all those properties which depend upon the number of gelatin molecules in solution increase with the proportion of gelatin salt formed, the maximum being reached when all the insoluble gelatin is transformed into soluble gelatin salt. This explains the parallelism between the curves for the bromine number of gelatin treated with HBr and the curves for the osmotic pressure of the solution. These molecular data must explain also the parallelism between the curves for viscosity and swelling with that of the bromine number.

The view taken in this paper that the osmotic pressure of protein solutions obeys the laws of classical physical chemistry is shared by one of the greatest authorities in this field, namely Sörensen.[9] Sörensen worked on egg albumin of a well defined composition which necessitated the investigation of the osmotic pressure of gelatin in the presence of ammonium sulfate. In spite of the great theoretical and experimental difficulties, which only a master like Sörensen could succeed in overcoming, he arrived at constant values for the osmotic pressure and the molecular weight of egg albumin. He states:

"The properties of colloidal solutions can be most efficiently inquired into by application, as far as possible, of the same views and methods as those generally applied to true solutions. . . .[10]

"Colloidal chemistry in the shape which has been given it by its energetic champion Wo. Ostwald offers, no doubt, to protein study a system organized with great talent, but exact experimental investigation has not been able to keep up with the systematic treatment, and therefore the value of the contents does not always correspond with the perfection of the system itself. Thus I disagree with Ostwald, who . . . warns us against a comparison of the circumstances in colloidal and real solutions. In the case of albumin solutions, and doubtless also several other typical emulsoid systems, such a comparison between the properties of the colloidal solution and those of a real solution is of the greatest significance for the right understanding of the character of the colloidal solution. Indeed, I think it is even possible to go one step further and to say that the study of real solutions may derive considerable advantage from the results which an exact research of well-defined protein solutions can give, the colloidal character of these permitting the use of research-methods—I refer especially to the use of

[9] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1917, xii.
[10] Sörensen, *Compt. rend. trav. Lab. Carlsberg*, 1917, xii, 369.

semi-permeable membranes—, which, when dealing with real solutions, can only be used in exceptional cases and under especial circumstances."[11]

SUMMARY.

1. The method of removing the excess of hydrobromic acid after it has had a chance to react chemically with gelatin has permitted us to measure the amount of Br in combination with the gelatin. It is shown that the curves representing the amount of bromine bound by the gelatin are approximately parallel with the curves for the osmotic pressure, the viscosity, and swelling of the gelatin solution. This proves that the curves for osmotic pressure are an unequivocal function of the number of gelatin bromide molecules formed under the influence of the acid. The cc. of 0.01 N Br in combination with 0.25 gm. of gelatin we call the bromine number.

2. The explanation of this influence of the acid on the physical properties of gelatin is based on the fact that gelatin is an amphoteric electrolyte, which at its isoelectric point is but sparingly soluble in water, while its transformation into a salt with a univalent anion like gelatin Br makes it soluble. The curve for the bromine number thus becomes at the same time the numerical expression for the number of gelatin molecules rendered soluble, and hence the curve for osmotic pressure must of necessity be parallel to the curve for the bromine number.

3. Volumetric analysis shows that gelatin treated previously with HBr is free from Br at the isoelectric point as well as on the more alkaline side from the isoelectric point (pH $\overline{>}$ 4.7) of gelatin. This is in harmony with the fact that gelatin (like any other amphoteric electrolyte) can dissociate on the alkaline side of its isoelectric point only as an anion. On the more acid side from the isoelectric point gelatin is found to be in combination with Br and the Br number rises with the pH.

4. When we titrate gelatin, treated previously with HBr but possessing a pH = 4.7, with NaOH we find that 25 cc. of a 1 per cent solution of isoelectric gelatin require about 5.25 to 5.5 cc. of 0.01 N NaOH for neutralization (with phenolphthalein as an indicator).

[11] Sörensen, *Compt. rend. trav. Lab. Carlsberg*, 1917, xii, 5–6.

This value which was found invariably is therefore a constant which we designate as "NaOH (isoelectric)." When we titrate 0.25 gm. of gelatin previously treated with HBr but possessing a pH<4.7 more than 5.5 cc. of 0.01 N NaOH are required for neutralization. We will designate this value of NaOH as "$(NaOH)_n$," where n represents the value of pH. If we designate the bromine number for the same pH as "Br_n" then we can show that the following equation is generally true: $(NaOH)_n = NaOH\ (isoelectric) + Br_n$. In other words, titration with NaOH of gelatin (previously treated with HBr) and being on the acid side of its isoelectric point results in the neutralization of the pure gelatin (NaOH isoelectric) with NaOH and besides in the neutralization of the HBr in combination with the gelatin. This HBr is set free as soon as through the addition of the NaOH the pH of the gelatin solution becomes equal to 4.7.

5. A comparison between the pH values and the bromine numbers found shows that over 90 per cent of the bromine or HBr found was in our experiments in combination with the gelatin.